The role of the melanocortin receptors in adrenal growth, development and stem cell maintenance

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Appendix A: Required statement of originality for inclusion in research degree theses

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Antibodies to POMC peptides provided by Dr A Bicknell, University of Reading

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Abstract

The adrenal gland is a highly dynamic organ with the ability to respond to changes in its environment with rapid changes in hormone production, and dramatic remodelling of its structure. Our understanding of the role of the melanocortin peptides in this process has been mostly restricted to the anterior pituitary hormone ACTH, via the melanocortin 2 receptor MC2R. The presence of additional melanocortin receptors and their antagonists has been demonstrated in rat and bovine adrenals, but the role of these in humans has not been well explored.

An intensive clinical and biochemical work-up of a single patient with ACTH-independent Cushing's syndrome and a gastrointestinal stromal tumour (GIST) was performed. The data presented are consistent with the possibility that her disease was caused by release of some bioactive molecule released from the GIST. We propose that alpha-MSH is a possible candidate for this molecule, on the basis that the GIST immuno-stained for alpha-MSH but not ACTH, that alpha-MSH but not ACTH was present in supernatant from a primary culture, and that alpha-MSH has the potential to stimulate cortisol production from adrenal cells. The precise mechanism for alpha-MSH secretion from the tumour is not fully elucidated, and further work is required to corroborate this hypothesis.

The patient had both pigmented skin and pigmented adrenal nodules, and we further demonstrated the presence of the alpha-MSH receptor MC1R was demonstrated with her excised adrenal gland. The pigment was identified as melanin, and we went on to show that same pattern in primary pigmented nodular adrenal disease, and to demonstrate that the zona reticularis in normal adrenal gland contains melanin, and has additional features in common with melanocytic tissues elsewhere in the body.

The role of alpha-MSH in normal adrenal function, and the possibility that melanin is also playing an important role, perhaps for its antioxidant properties, is an exciting area for future study.

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List of Abbreviations

11βHSD2	11β hydroxysteroid dehydrogenase
17OHP	17 hydroxy progesterone
17βHSD	17β hydroxysteroid dehydrogenase
5-HT	5 hydroxy tryptophan
ACTH	Adrenocorticotrophic hormone
ADM	Adrenomedullin
AGP	Adrenogonadal primordium
AGRP	Agouti related peptide
AHC	Adrenal hypoplasia congenita
AII	Angiotensin II
AIMAH	ACTH independent macronodular adrenal hyperplasia
AP	Alkaline phosphatase
AP-1	Activator protein 1 transcription factor
ARMC5	armadillo repeat containing 5
AsP	Adrenal secretory protease
AT1	Angiotensin receptor
b5	Cytochrome b5
BLAST	Basic local alignment search tool
BRAF	B-Rapidly Accelerated Fibrosarcoma
CASH	Cortical androgen stimulating factor
CBX1	Chromobox protein homolog 1
CD117	Tyrosine protein kinase kit (Gene)
CDK	Cyclin dependent kinase
CDKN1C	Cyclin dependent kinase N1C (p57 kip2)
Cited2	CBP/p300-interacting transactivator with ED-rich tail 2
CNC	Carney Complex
CNS	Central nervous system
COMT	catechol-O-methyltransferase
CPE	Carboxypeptidase E
CREB	cAMP response element binding protein
CRH	Corticotropin releasing hormone
CYP11A1	Cytochrome p450 side chain cleavage (gene)
CYP11B1	11-β hydroxylase (gene)
CYP11B2	Aldosterone synthase (gene)
CYP17A1	17 alpha hydroxylase and 17,20 lyase (gene)
CYP21A1	21-hydroxylase (gene)
DAB	Diaminobenzidene
DAd	Definitive adrenal
Dax1	dosage sensitive sex reversal adrenal hypoplasia congenita

	(AHC) critical region on the X chromosome
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
Dhh	Desert hedgehog
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DOC	Deoxycorticosterone
DOG-1	Discovered on GIST 1
dpc	Days post conception
DTT	Dithiothreitol
DZ	Definitive zone
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ERK	Extracellular signal related kinases
FAd	Fetal adrenal
FBS	Fetal bovine serum
FdR	Ferredoxin reductase
FDx	Ferredoxin
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FoxD1	Forkhead box D1
FoxD2	Forkhead box D2
FZ	Fetal zone
GCM	GIST conditioned media (Cushing's patient)
GH	Growth hormone
GIST	Gastrointestinal stromal tumour
Gli1	Glioma associated oncogene 1
Gli2	Glioma associated oncogene 2
Gli3	Glioma associated oncogene 3
GnRH	Gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
H&E	Haematoxylin and Eosin
HDL	High density lipoprotein
Hh	Hedgehog
HMB-45	Human melanoma black antigen 45
HPA	Hypothalamic adrenal axis
HRP	Horseradish peroxidase
HSD	Hydroxysteroid dehydrogenase
HU	Hounsfield Units

IFN-γ	Interferon gamma
IGF-1	Insulin like growth factor 1
IGF2	Insulin like growth factor 2
IGFR1	Insulin like growth factor receptor 1
IHC	Immunohistochemistry
Ihh	Indian hedgehog
IL-1	Interleukin 1
IL-6	Interleukin 6
IMM	Inner mitochondrial membrane
Insr	Insulin receptor (gene)
IVC	Inferior vena cava
iZU	inner undifferentiated zone
JP	Joining peptide
LDL	Low density lipoprotein
LEF	Lymphoid enhancer binding factor 1
MAPK	Mitogen activated protein kinase
MART-1	Melanoma antigen A
MC1R	Melanocortin receptor 1
MC2R	Melanocortin receptor 2
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MC5R	Melanocortin receptor 5
MEN	Multiple endocrine neoplasia
MITF	Microophthalmia induced transcription factor
MR	Mineralocorticoid receptor
N-POC	N-terminal pro-opiomelanocortin
N-POMC	N-terminal pro-opiomelanocortin
NADPH	Nicotinamide adenine dinucleotide phosphate - reduced form
NAT	N-acetyl transferase
NCM	GIST conditioned media (Control patient)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Oct3/4m	Octamer binding transcription factor 3/4
Odd1	Odd skipped related 1
oZU	outer undifferentiated zone
p450c11AS	Aldsterone synthase
p450c17	17 alpha hydroxylase and 17,20 lyase
p450c21	21-hydroxylase
p450scc	Cytochrome p450 side chain cleavage
PACAP	Pituitary adenylate cyclase-activating peptide
PAM	Peptidyl glycine alpha-amidating mono-oxygenase
PAS	Periodic Acid Schiff
Pbx1	Pre-B-cell leukemia transcription factor 1

PC1/3	Proprotein convertase 1/3
PC2	Proprotein converase 2
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDE11A	Phosphodiesterase 11A
PDE8B	Phosphodiesterase 8B
PDGFRA	Platelet derived growth factor receptor A
PI3K	Phosphoinositide
PKA	Protein kinase A
PKA	Protein kinase A
Pmel17	Premelanosome protein 17
PNMT	phenylethanolamine N-methyltransferase
Pod1	Podocyte expressed 1
POMC	Pro-opiomelanocortin
POR	Cytochrome p450 reductase
Ppi	Pyrophosphate
PPNAD	Primary pigmented nodular adrenal disease
PRCP	Lysosomal pro-X carboxypeptidase
PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit
PTCH1	Patched 1
qPCR	quantitative PCR
RAS	Renin-angiotensin-aldosterone system
RhoA	Ras homolog gene family member A
RNA	Ribonucleic acid
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
RT	Room temperature
RT-PCR	Reverse transcription PCR
SA	Streptavidin
Sall1	Sal-like 1
SDH	Succinate dehydrogenase
SF-1	Steroidogenic factor 1
Shh	Sonic Hedgehog
Smo	Smoothened
SR-B1	Scavenger class B receptor member 1
StAR	Steroidogenic acute regulatory protein
SULT2A1	Sulphotransferase 2A1
TCF	T cell factor
TESPA	3-triethoxysilylpropylamine
THP-1	A human acute monocytic leukaemia cell line
TMA	Tissue microarray
TMB	Tetramethyl benzidine
TNF-α	Tumor necrosis factor alpha

TZ	Transitional zone
UV	Ultraviolet
VIP	Vasoactive intestinal peptide
Wnt	Wingless-related mouse mammary tumour virus integration site
wpc	Weeks post conception
WT1	Wilms tumour gene 1
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZN	Ziehl-Neelsen
ZR	Zona reticularis
ZU	Zona intermedia
α-MSH	alpha melanocyte stimulating hormone
β-LPH	beta lipotrophin
β-MSH	beta melanocyte stimulating hormone
γ-LPH	gamma lipotrophin
γ-MSH	gamma melanocyte stimulating hormone

Chapter 1 Introduction

1.1 The Adrenal Gland

The adrenal gland is a vital mediator of an organism's response to stress, and a regulator of immune function, blood pressure and energy homeostasis. It focuses multiple endocrine systems within a single organ: the hypothalamic-pituitary adrenal (HPA), the renin-angiotensin-aldosterone system (RAS), and the sympathoadrenal. This close anatomical proximity is no coincidence, and in recent times we have come to understand more about the communication between these systems (Haase et al., 2011; Schinner & Bornstein, 2005). The adrenal gland has a remarkable capacity for regeneration and remodelling (Bland et al., 2003; Brennan et al., 2008), and recent insights into its developmental origins (Wood & Hammer, G. D., 2011) have aided our understanding of the functioning of the gland in normal physiology and disease.

I will describe the anatomy of the gland, and how that structure relates to its function as an endocrine organ both at a macroscopic and microscopic level. I will explore molecular mechanisms underpinning its embryology and post-natal growth and remodelling, and the biochemistry of hormone production. I will discuss the manifold internal and external stimuli that are employed to control these processes, with a focus on glucocorticoid production.

1.1.1 History

The adrenal gland was described in antiquity by Galen, but only recognised as a discrete organ by Eustachi in 1563 (Eustachi, 1564). 19th century advances in lens technology allowed Von Kolliker to define its microscopic anatomy (Kölliker & Huxley, 1854), and in 1855 the clinical consequences of failure of "the supra-renal capsules" was described by Thomas Addison (Addison, 1855). Charles Brown-Sequard postulated the existence of hormones fifty years before Bayliss and Starling identified secretin, and in 1856 performed adrenalectomies on several different species of animal which resulted in their death, confirming the organ's vital role (Brown-Séquard, 1856).

In 1886 Felix Frankel was the first to describe a phaeochromocytoma, a tumour derived from the catecholamine secreting cells of the adrenal medulla (Fraenkel, 1886). The pressor effects of adrenal extract were shown ten years later by Oliver and Schafer (Oliver & Schäfer, 1895) before adrenaline was purified by John Abel in 1899 (Abel, 1901). Harvey Cushing described the consequences of severe glucocorticoid excess in 1910 (Cushing, 1912), but it was not until the 1930s that the hormones of the adrenal cortex were identified by Edward Kendall, Tadeus Reichstein and Philip Hench, winning them the Nobel Prize for physiology in 1950 (Hench & Kendall, 1949).

The existence of adrenal steroids with pure mineralocorticoid activity was a source of controversy until it was formally determined that electrocortin was secreted from the zona glomerulosa under provocation from a low salt diet or potassium loading (Deane et al., 1948). Simpson and Tait isolated electrocortin from bovine adrenals, and the structure of the hormone - now renamed aldosterone - was reported in 1953 (Grundy et al., 1952; Simpson, S. A. et al., 1953).

1.2 The Anatomy of the Adrenal Gland

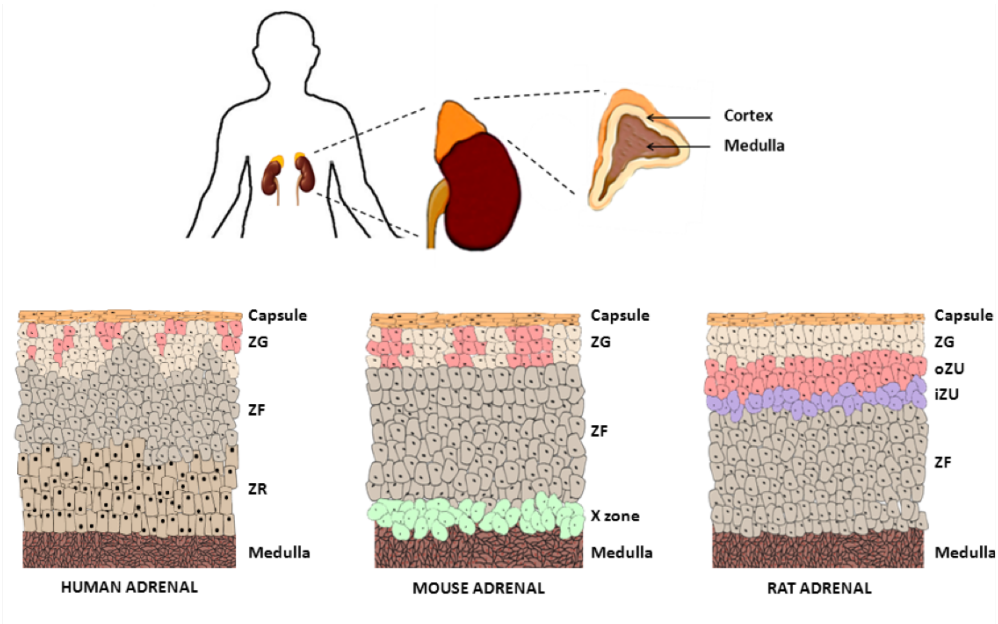

Figure 1.1 Anatomy and structure of the adrenal gland
(A) **The adrenal glands are located at the upper poles of the kidneys. (B) Zonation differs between species. The human gland has three recognisable cortical zones, the ZG, ZF and ZR, surrounding the medulla. The location of Shh-expressing cells in each of the species is shown in pink, in the rat**

this takes the form of an undifferentiated ZU between ZG and ZG. The X zone in the mouse forms around the time of birth, and remains until puberty in males or first pregnancy in females. From Yates et al (Yates et al., 2013).

1.2.1 Macroscopic Anatomy

The human adrenal gland (Figure 1.1) is located anterior and superior to the upper part of the kidney, within its own compartment of renal fascia (Gray et al., 2005). The adult human glands weigh approximately 8-10g and are approximately 90% cortex. The right gland is pyramidal in shape, and the left more crescentic. A thick, collagenous capsule composed of fibroblasts and myofibroblasts extends trabeculae deep into the cortex, and the latter completely encloses the medulla, except at the hilum. Veins and lymphatic vessels leave at the hila, but arteries and nerves enter the gland at multiple sites.

1.2.2 Adrenal Histology

Underneath the capsule, the cortex is subdivided into three main zones, first described and named by Arnold in 1852 on the basis of morphological appearance (Arnold, 1886)

The zona glomerulosa (ZG) is composed of small, narrow, polyhedral cells in rounded clusters, with deep staining nuclei and basophilic cytoplasm, and a few lipid droplets. They contain abundant smooth ER, typical of steroidogenic cells, and their mitochondria are characterised by lamelliform (shelf-like) cristae (Belloni et al., 1987). The ZG makes up around 15% of the cortex depending on the prevailing sodium intake of the animal. The main hormone produced is the mineralocorticoid aldosterone.

The zona fasciculata (ZF) cells are larger than the ZG and due to their paler staining properties are known as clear cells (Wheater et al., 2013). They are laid out in columns two cells wide, parallel to fenestrated sinusoids, and contain abundant lipid and smooth ER. In humans some of these columns reach past the ZG clusters to the capsule (Boulkroun et al., 2010). The cristae of ZF mitochondria are tubulovesicular in shape (Robba et al., 1980; Belloni et al., 1987). The ZF predominantly secretes glucocorticoid: cortisol in humans, corticosterone in rodents.

The zona reticularis (ZR) are a branching network of smaller, compact cells with numerous smooth ER and multiple lysosomes containing brown lipofuscin pigment. In the context of chronic ACTH stimulation there is gradual lipid depletion from the clear

cells and they are said to become more compact-like in appearance(Hornsby, 2002). The ZR also produces glucocorticoid, and additionally in humans and higher primates secretes adrenal androgens - androstenedione and dehydroepiandrosterone (DHEA) and its sulphate DHEAS (Parker, L. N. & Odell, 1980).

The inner mass of the gland is the medulla, with little connective tissue separating it from the cortex. This leaves the cells in direct contact, allowing crosstalk between the two adrenal departments (Schinner & Bornstein, 2005). The medulla is composed of groups and columns of chromaffin cells, although there are also smaller islands of these scattered throughout the cortex (Gallo-Payet et al., 1987). The cells are large, with large nuclei, and contain fine cytoplasmic granules which stain brown with chromium salts (and are therefore named phaeochromocytes) (Sjostrand & Wetztein, 1956; Bander, 1951). Catecholamine hormones are packaged within the granules and released in response to stimulation from sympathetic nerve terminals, which contact the cells on the opposite side to venous sinusoids (Bulbring & Burn, 1949). 80% of the medulla cells secrete adrenaline, and 20% noradrenaline, with a few dopaminergic cells (Wong, 2003). They are functionally equivalent to postganglionic sympathetic nerves.

The most studied mammalian species are humans, mice and rats, and they demonstrate a few notable differences in adrenal zonation (Figure 1.1). The ZG is a continuous zone beneath the capsules in rats, and is separated from the ZF by a sudanophobic zone three to 4 cells thick known variously as the zona intermedia or undifferentiated zone (ZU), with mitochondria similar to ZF (Cater & Lever, 1954; Mitani et al., 2003). The ZR in mice and rats is often difficult to identify histologically (Tanaka, S. & Matsuzawa, 1995), and does not express CYP17A1, so these species do not produce adrenal androgens: the ZR and ZF are therefore thought to be functionally equivalent (Endoh et al., 1996). In mice an additional inner X-zone adjacent to the medulla is present, but which involutes at puberty in the male or first pregnancy in the female. The function of this zone is obscure, and while it does not express 3β-hydroxysteroid dehydrogenase, it does express 20α-hydroxysteroid dehydrogenase, enabling catabolism of progesterone and 11-deoxycorticosterone (Hershkovitz et al., 2007). There is a wide variation in the appearance of the ZR between species, with some expressing little functional tissue at all (Suto, 2012).

1.2.3 Blood supply

Arterial blood supply is abundant with arteries branching freely before up to 60 vessels penetrate the capsule - there are usually 6-8 superior adrenal arteries branching from the inferior phrenic artery, and sometimes a middle adrenal artery direct from the aorta, and an inferior adrenal artery from the renal artery (Dobbie & Symington, 1966). On entering the gland these vessels form a subcapsular capillary plexus that gives rise to fenestrated sinusoids which pass through the gland before forming a further plexus in the zona reticularis. There are also direct branches to the medulla from the subcapsular plexus in all mammalian species (Coupland & Selby, 1976). This complex vascular arrangement ensures that most cells in the adrenal are only one to two cells away from a vascular endothelial cell, with efficient delivery of adrenal hormones to the blood stream(Vinson et al., 1985). The human adrenal vasculature is represented in figure 1.2.

Venules pass between the chromaffin cells and enter the medullary veins between bundles of smooth muscle fibres, merging into a single adrenal vein at the hilum (Idelman, 1970). On the right this is usually directly to the inferior vena cava (IVC) with a longer vein on the left into the renal vein (Dobbie & Symington, 1966). There are lymphatic plexus deep to the capsule and in the medulla which drain through the hilum to para-aortic nodes (Merklin, 1966).

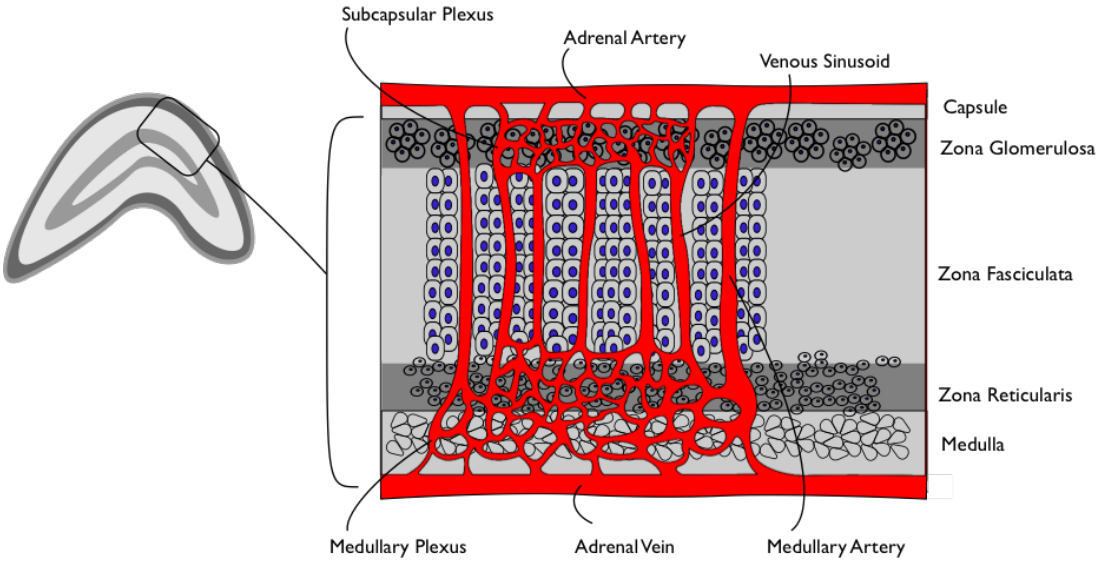

Figure 1.2 Blood supply of the human adrenal gland
Vessels entering the gland form a subcapsular capillary plexus that gives rise to fenestrated sinusoids which pass through the gland to reach a further plexus in the ZR. Most cells in the adrenal

gland are only one to two cells away from a vascular endothelial cell, allowing for efficient delivery of hormones to the blood stream.

1.2.4 Nerve Supply

Relative to its size, the adrenal gland has a more substantial autonomic nerve supply than any other organ (Parker, T. L. et al., 1993). Myelinated preganglionic sympathetic fibres from splanchnic nerves originating from the interomediolateral cell column or lateral horn of T10-11 synapse directly on medullary chromaffin cells (Idelman, 1970).

Early descriptions of adrenal microanatomy suggested that there was no direct innervation to the cortex (Elliott, 1913; Hollinshead, 1936), but this was challenged by Alpert (Alpert, 1931) and confirmed by Unsicker's observations in the early 1970s of nerve fibres synapsing directly on to cortical cells in the pig and rat adrenal glands (Unsicker, 1971). There is also post-ganglionic sympathetic supply to blood vessels within the cortex, which is thought to have an element of ACTH control attached (Holzwarth et al., 1987), and is thought to be largely mediated by neuropeptides as we will discuss (Breslow et al., 1987).

1.3 Adrenocortical physiology

1.3.1 Functional zonation of the adrenal cortex

The main hormonal products of the adrenal gland are corticosteroids, and medullary catecholamines. Functional zonation of the adrenal cortex allows mineralocorticoid, glucocorticoid and adrenal androgen secretion to be regulated independently of each other. This is achieved in part through the differential expression of steroidogenic enzymes and cofactors in the different zones, and distinct cell surface receptors (Miller & Auchus, 2011).

In all cortical cells steroid production begins with cholesterol uptake, there is initial trafficking to the inner mitochondrial membrane (IMM), then conversion to pregnenolone by the cytochrome p450 enzyme p450scc (side chain cleavage). The main source of cholesterol is plasma low density lipoprotein (LDL) with a lesser contribution from intracellular acetyl CoA (Mason & Rainey, 1987). Rodents obtain cholesterol primarily

from HDL using a SR-B1 selective pathway (Valacchi et al., 2011). In a zone dependent manner ACTH, angiotensin II (AII) and increasing potassium ion (K^+) concentrations all increase cholesterol availability by increasing expression and activity of LDL receptors, stimulating HMG CoA reductase and hormone sensitive lipase (Kraemer, 2007). The steroidogenic acute regulatory protein (StAR) controls trafficking of cholesterol to the IMM, and this is also up-regulated and phosphorylated following ACTH and AII/K^+ stimulation (Mathieu et al., 2002; Murcia et al., 2006). In this way steroid hormone secretion can be induced within minutes. Cholesterol trafficking is favoured by the tubulovesicular mitochondria, as in the ZF and ZR.

The steroidogenic enzyme pathway is illustrated in figure 1.3. The enzymes employed belong to the cytochrome p450 and hydroxy-steroid dehydrogenase (HSD) classes. Cytochrome p450 enzymes use electrons derived from NADPH to facilitate their catalytic actions (Gonzalez, 1988). HSD reactions convert hydroxysteroids to ketosteroids using nicotinamide cofactors without intermediary proteins, but unlike the p450 enzymes, different isoforms may catalyse the reverse reaction (Agarwal & Auchus, 2005). The intermediate products of these enzymes shuttle back and forth between smooth endoplasmic reticulum and inner mitochondrial membrane.

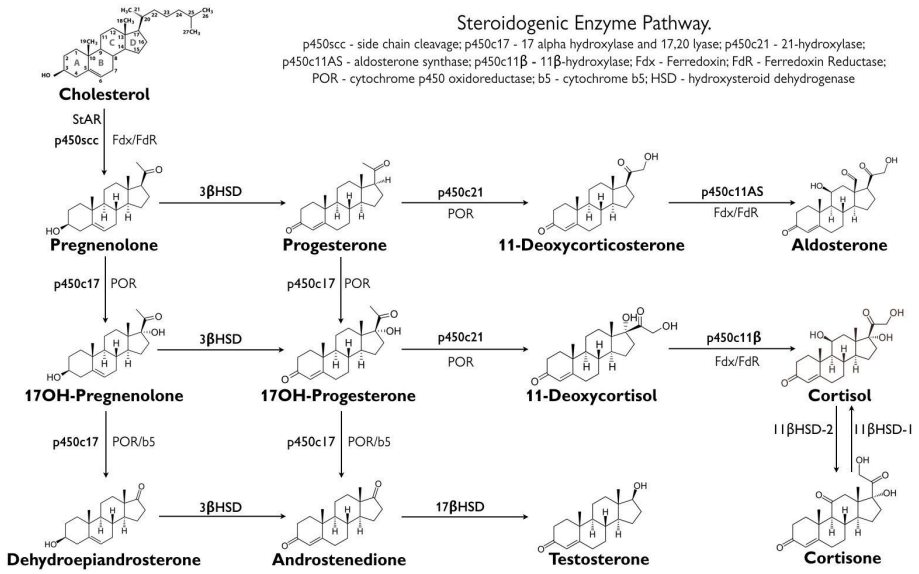

Figure 1.3 The steroidogenic enzyme pathway in humans

1.3.2 Cytochrome p450 enzymes

Cytochrome p450 enzymes comprise the mitochondrial type I and the microsomal type II enzymes. They use different cofactors as discussed below.

Ferredoxin (Fdx) and **ferredoxin reductase (FdR)** are a complex of enzymes on the IMM which transfer electrons from NADPH to p450scc, with a pair being required for each of the three sequential reactions catalysed by this enzyme (Miller, 2005) . Fdx forms a complex with FdR, then dissociates to form a similar complex with p450scc. Regulation of FdX/FdR expression does not seem to be a point of hormonal regulation of steroidogenesis(Ziegler et al., 1999). FdX/FdR are also the electron donors for p450c11β and p450c11AS.

The type II enzymes (**CYP17A1** and **CYP21A1**) receive their electrons from NADPH via **P450 oxidoreductase** (POR). POR always donates the first electron, but the second may be provided by cytochrome b5 (Storbeck et al., 2013). The availability of electrons from POR is a rate-limiting step.

p450scc (protein), **CYP11A1** (gene): The rate limiting step in steroidogenesis, present in all 3 zones; this catalyses three reactions – hydroxylation at C-20, C-22, and cleavage of the amine side chain(Koritz & Kumar, 1970). The pregnenolone generated is trafficked back out of the mitochondria.

p450c17 (CYP17A1): This enzyme catalyses two reactions - 17 alpha-hydroxylation, and 17,20 lyase - which were originally thought to be the actions of two separate enzymes. The two activities can be controlled separately to a degree by availability of cofactors. The 17,20 lyase activity is favoured in the ZR by increased expression of POR and cytochrome b5 , with increased androgen secretion as a consequence (Endoh et al., 1996). The activity of CYP17A1 is also influenced by phosphorylation, which seems to favour the 17,20 lyase activity (Miller, 2005). CYP17A1 is not expressed in adult rats and mice, so the ZR of these animals does not synthesise adrenal androgens, but the fetal zone of the developing adrenal in these species does express the enzyme, and therefore is able to produce large amounts of DHEA. This is thought to be the precursor for placental oestrogen production (Kaludjerovic et al., 2012)

p450c21 (CYP21A1): The 21 alpha hydroxylase enzyme that generates 11-deoxycorticosterone (DOC) from progesterone, and 11-deoxycortisol from 17α-hydroxyprogesterone (17OHP). The CYP21 gene locus is highly complex, with both a functional CYP21A1 gene and CYP21A1P pseudogene within the HLA locus on chromosome 6. The HLA locus is highly recombinogenic, and exchange of DNA between the gene and pseudogene during cell division is common (Nebert et al., 2013). Gene conversion events can lead to some or all of the pseudogene replacing the corresponding area of CYP21A1, with decreased expression or reduced activity of the p450c21 protein. 21-hydroxylase deficiency is therefore on of the most common autosomal recessive disorders affecting 1 in 20000 live births (Auchus, 2010). In the absence of enzyme activity, the inability to convert progesterone to DOC results in aldosterone deficiency, and the inability to convert 17OHP to 11-deoxycortisol results in cortisol deficiency. At its most severe this causes hypoglycaemia, hyponatraemia, hyperkalaemia and acidosis, with hypotension and cardiovascular collapse. The absence of cortisol delivery to the medulla results in lower adrenaline levels, compounding the hypotension and hypoglycaemia (Merke et al., 2000). Increased shuttling of intermediates to adrenal androgens causes variable amounts of virilisation depending on the specific abnormality.

p450c11β (CYP11B1) and **p450c11AS (CYP11B2):** These closely related enzymes catalyse the final steps in the synthesis of glucocorticoids and mineralocorticoids, following trafficking of DOC or deoxycortisol back to the IMM (White, P. C. & Pascoe, 1992). p450c11β has a single 11-hydroxylation activity and is expressed in the ZF; p450c11AS can also catalyse 18-hydroxylation and 18 methyl-oxidation and its expression in the ZG restricts aldosterone production to this layer. An unequal crossing over of these genes can result in glucocorticoid-remediable hyperaldosteronism, when a hybrid gene allows aldosterone synthesis in the ZF, stimulated by ACTH (Salti et al., 1969; Lifton et al., 1992). The condition is named because treatment with glucocorticoid will have a negative feedback on the pituitary and reduce the ACTH drive to the ZG. A lack of CYP17A1 expression in rats and mice means that DOC is the substrate for both p450c11β as well as p450c11AS and therefore the main glucocorticoid produced in these species is corticosterone (Miller & Auchus, 2011).

1.3.3 Hydroxy-steroid dehydrogenase enzymes

3βHSD is common to all of the steroidogenic cells(Mason et al., 1997). It converts the C-3 hydroxyl to a keto group, and isomerises the double bond from the B to the A ring. The type 2 isoform is predominant in the adrenal gland and gonad. Low levels in the ZR impair conversion of DHEA to androstenedione, ensuring that DHEA is the most abundant adrenal androgen (Rainey et al., 2002). DHEA can be sulphated by sulphotransferase SULT2A1 to the inactive DHEA-S (Arlt et al., 1999).

17βHSD: Isoforms of this are important in the gonad and the periphery in generating testosterone and interconverting oestrogens. Small amounts of testosterone in the ZR are probably a result of local production of 17βHSD5 (Nakamura et al., 2009).

11βHSD1 and 11βHSD2: Although not expressed in the adrenal, the ability of the two isoforms of this enzyme to interconvert cortisol and its inactive metabolite cortisone is vital to regulating the actions of both cortisol and aldosterone. 11βHSD2 prevents cortisol from activating the mineralocorticoid receptor in the kidney (Funder et al., 1988), while 11βHSD1 allows amplification of the cortisol signal in liver, muscle and nervous tissue (Seckl, 2004)

1.3.4 Mineralocorticoids

The principal mineralocorticoid is aldosterone, secreted in amounts 3 orders of magnitude less than cortisol. DOC has mineralocorticoid activity but this is not physiologically significant (Vinson & Coghlan, 2010). ZG cells are distinguished by expression of the type I angiotensin II receptor (AT1), aldosterone synthase, low levels of p450c17, and no expression of p450c11β. ZG cells do express receptors for ACTH but the main regulators of secretion are angiotensin II (Ang II) (Ganong et al., 1962) and small increases in the extracellular potassium concentration (Bing & Schulster, 1977). Angiotensin II levels increase because of volume depletion causing renin release in the kidney, but sodium depletion does not seem to cause aldosterone release through this mechanism (Okubo et al., 1997). Aldosterone release is inhibited by somatostatin, heparin, atrial natriuretic peptide and dopamine (Boscaro et al., 1982; Chartier et al., 1984). Additional control mechanisms will be discussed in more detail later.

Binding of Ang II to the G-protein coupled AT1 receptor activates multiple downstream signalling pathways (Bird et al., 1993). Phospholipase C is the best characterised second messenger, causing an increase in intracellular calcium (Wojcikiewicz & Nahorski, 1993). The ZG cell membrane is depolarised by increasing extracellular concentrations of potassium, leading to calcium influx. In the short term StAR activity is increased (minutes to hours), while in the longer term (hours to days) CYP11B2 is upregulated (Pezzi et al., 1997; Nogueira et al., 2009). ZG cell proliferation is upregulated by Ang II mediated up-regulation of the mitogen-activated protein kinase pathway (McNeill et al., 1998).

The classical effects of aldosterone are mediated by binding to the mineralocorticoid receptor (MR) in the cytosol (Funder et al., 1972). The MR is distributed widely across a range of tissues, including the kidney, cardiomyocytes and vascular endothelium (Cat & Jaisser, 2012). It is a member of a steroid receptor superfamily that includes the glucocorticoid receptor and the androgen receptor (Arriza et al., 1987). Ligand binding induces dimerisation and dissociation from chaperone proteins, including hsp 90. The bound receptor translocates to the nucleus and is recruited to target genes by direct DNA binding or by associating with other DNA-bound transcription factors. There are aldosterone-induced effects on the vascular system which take place over a time course which is too rapid to be accounted for by transcriptional mechanisms alone. For instance, aldosterone injection in healthy human subjects was associated with an increase in vascular resistance within the first 10 minutes (Briet & Schiffrin, 2013). The 7-transmembrane spanning, G-protein coupled receptor GPR30 has been suggested as one receptor involved in these non-genomic effects of aldosterone (Gros et al., 2011). Some of these rapid effects are abrogated by the MR antagonist eplerenone, and may be mediated by the MR binding additional receptors itself (Funder, 2010).

The MR binds cortisol and aldosterone with equal affinity, and it is widely held that cortisol, present in 100-fold excess of aldosterone, is inactivated by the action of 11βHSD-2 in the kidney. However, even in tissues with a plentiful supply of this enzyme, clearance of active cortisol is rarely complete, remaining at 10 times the level of aldosterone (Funder, 1996). The mechanism by which MR occupied by cortisol is held inactive is yet to be fully elucidated, but may be a consequence of the prevailing preponderance of reduced NAD^+ in these cells preventing activation of the MR which has bound cortisol (Funder, 2010).

Aldosterone induces renal retention of sodium, by upregulating epithelial sodium channels (ENaC) in the distal tubules(Bastl & Hayslett, 1992; Bubien, 2010). Although widely held to be the mechanism by which hyperaldosteronism increases blood pressure, this is not the sole mechanism - direct aldosterone actions on the vasculature and the CNS are also important (Moura & Worcel, 1984). Oxidative stress upregulates the expression of the MR in cardiac cells, where it induces cardiac remodelling and fibrosis (Favre et al., 2011). Aldosterone itself can promote inflammation and oxidative stress, and contribute to impaired insulin signalling (Luther et al., 2011), while it induces swelling and stiffening of vascular epithelial cells and counteracts their ability to trigger vasodilatation (Oberleithner et al., 2004). This pro-inflammatory role is in contrast to the anti-inflammatory effects of glucocorticoid signalling.

1.3.5 Glucocorticoids

Glucocorticoid production is under the control of the hypothalamic-pituitary adrenal (HPA) axis. Upon activation, parvocellular neurons of the hypothalamic paraventricular nucleus synthesise and secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal circulation (Chrousos, 1995; Sapolsky et al., 2000). CRH binding to the CRH receptor on the corticotroph cell surface leads to ACTH release into the systemic circulation (Carrasco & Van de Kar, 2003). AVP has a synergistic role but does not stimulate ACTH release alone (Chrousos, 1995). CRH neurons projecting directly on to pro-opiomelanocortin (POMC) containing neurons in the hypothalamic arcuate nucleus can also stimulate local ACTH production, by inducing POMC cleavage (Pritchard & White, 2007). Cortisol levels are increased in line with the circadian rhythm of ACTH secretion, and in response to physical stresses such as hypotension, hypoglycaemia and fever which activate the HPA axis (Engeland et al., 1977).

ACTH binds to the melanocortin-2 receptor (MC2R), a 7-transmembrane G-protein coupled receptor (GPCR) which is expressed primarily in the ZF and activates cAMP-mediated downstream signalling pathways (Chida et al., 2007). The immediate effects are mediated through the activation of cAMP-dependent protein kinase A (PKA) phosphorylating and activating cholesteryl ester hydrolases and StAR, and bring about rapid changes in cholesterol trafficking. Chronic ACTH stimulation (hours to days) leads to increased expression of p450scc, p450c17, p450c21, p450c11β and ferredoxin (Sewer

et al., 2007; Waterman & Bischof, 1996). Adrenal blood flow increases (Vinson et al., 1985), and the ZF layer hypertrophies, although the specific role of ACTH in this process is questioned as we shall see. ACTH fails to induce high levels of DHEA secretion from the ZF due to the low levels of cytochrome b5 in this zone. Additional factors modulating steroid output including locally produced cytokines, neuropeptides, and autonomic innervation of the gland will be considered later (Mikhaylova et al., 2007; Tkachenko et al., 2011).

The glucocorticoid receptor is found in almost all cell types, and ligand binding is essentially identical to MR binding (Arriza et al., 1987). Receptor activation generally results in an inhibition of DNA synthesis (Santos et al., 2011). The effects of cortisol excess are well characterised, but the actual physiological role for the hormone is less clear cut, being predominantly a permissive one at basal levels. Glucocorticoids are named for their effect in modifying glucose metabolism - they stimulate gluconeogenesis and antagonise insulin - but whether this is an important homeostatic mechanism in the day to day life of normal individuals is debated (Vinson, 2009). The importance of cortisol production in response to starvation-induced stress in providing substrates for gluconeogenesis from muscle breakdown, for instance, is likely to have been more important from an evolutionary perspective (Nakamura et al., 2016).

In the cardiovascular system cortisol increases the transcription of receptors for angiotensin II, epinephrine and norepinephrine, supporting a role in maintaining blood pressure (Sakaue & Hoffman, 1991). A role for inhibiting inflammation through negatively regulating signalling pathways controlled by NFκB and AP-1 transacting factors, and by down-regulating anti-inflammatory cytokine gene expression (IL-1-6, IL-12, IFN-γ, TNF-α), is hypothesised to be more significant (Franchimont, 2004). The pro-inflammatory effects of MR activation, which can be stimulated by cortisol, would appear to support this contention. The CNS effects of increasing appetite and paranoia under stress have a theoretical evolutionary benefit, and are commonly observed in cortisol excess (Bertagna et al., 2009).

1.3.6 Adrenal Androgens

Adrenal androgens are the most abundant steroids secreted from the adrenal gland (>20 mg per day), principally DHEA and DHEAS, with smaller amounts of androstenedione

(A4) and very small amounts of testosterone. Maturation of the zona reticularis occurs at adrenarche in humans, gorillas and chimpanzees, usually at aged 8-10 in the human (Hui et al., 2009). ACTH has a permissive role, and a cortical androgen stimulating factor (CASH) has long been sought, but the stimulus for ZR maturation is thought likely to be adrenal in origin (Hornsby, 2002). Roles for the GH/IGF1 axis, or oestrogens produced by aromatase in the medulla, may be important (Belgorosky et al., 2008). The physical consequences of ZR maturation at adrenarche are the development of pubic hair, with a possible CNS role for DHEA in fine-tuning of the GnRH pulse generator to signal puberty. Pubertal development per se is not affected, nor is the individual's final height, but premature adrenarche is associated with later metabolic disease and polycystic ovarian syndrome (Idkowiak et al., 2011).

The key feature of the ZR is low levels of expression of 3β-HSD (Sasano et al., 1990; Gell et al., 1996) The enzymes p450c17, POR and cytochrome b5 are up-regulated at adrenarche, favouring the 17,20 lyase activity and therefore androgen production (Miller et al., 1997). The mass of the ZR increases, and 3βHSD1 levels decrease, so more DHEA is produced than A4. The MC2R is expressed in the ZR, but cortisol production is restricted by low levels of p450c21 and p450c11β1. As DHEA accumulates, there is conversion to A4, and to testosterone in very small amounts. SULT2A1, the sulphotransferase which produces DHEAS, is up-regulated at adrenarche, but whether DHEAS is an active endocrine hormone is not clear (Suzuki, T. et al., 2000). The affinity of DHEA for the androgen receptor is low until peripheral conversion to testosterone and dihydrotestosterone (Chen, F., 2005). As mentioned previously, the lack of CYP17 expression in rat and mouse means that adrenal androgens are not produced in these animals.

1.3.7 Catecholamines

Catecholamines are packaged with chromogranins and a variety of neuropeptides within the dense granules of chromaffin cells. The chromogranins have a role in processing, storage and release of the contents of the granules (Louthan, 2011).

Tyrosine is converted to dopamine by the successive actions of tyrosine hydroxylase and dopa-decarboxylase (Fukami et al., 1990). Dopamine β-hydroxylase converts this to noradrenaline, which is modified in the cytoplasm to adrenaline under the action of

phenylethanolamine N-methyltransferase (PNMT) in adrenergic chromaffin cells, before being taken up into another storage vesicle. Catecholamine half-life in the circulation is only 10-100 seconds, before being recovered by sympathetic nerves and chromaffin cells and metabolised to metanephrines by the action of catechol-O-methyltransferase (COMT) (De Diego et al., 2007). In 20% of chromaffin cells in humans PNMT is not expressed, and these cells therefore secrete noradrenaline (Wong, 2003).

Catecholamine release is stimulated by acetylcholine release from the preganglionic sympathetic nerves, but there is a basal secretion even without neural input (De Diego et al., 2007). The pathways inducing adrenaline and noradrenaline release are different, with preferential noradrenaline release in response to haemorrhage, and adrenaline in response to hypoglycaemia (De Diego et al., 2007). The "fight or flight" response to severe stress, originally described by W B Cannon (Cannon, W. B., 1915), is due to a 60-fold increase in catecholamine secretion and is associated with increases in heart rate, metabolic rate and BP, glucose mobilised from liver and muscle, and dilatation of bronchioles and pupils.

The commonly held view of the adrenal gland as containing two anatomically and functionally distinct zones of cortex and medulla has been increasingly challenged, with it becoming clear that there is a great deal of cross-talk between the two. (Haase et al., 2011; Ehrhart-Bornstein & Bornstein, 2008). The close contact between cortex and medulla without an intervening connective tissue layer provide the morphological pre-requisite (Schinner & Bornstein, 2005), and as described previously, the structure of the adrenal blood supply ensures that the medulla is exposed to high levels of cortisol. This has been shown to be a requirement for the expression of PNMT and biosynthesis of adrenaline in medullary chromaffin cells (Wurtman & Axelrod, 1965). Conversely, deletion of the tyrosine hydroxylase gene in mice leading to catecholamine deficiency also results reduced steroid production from the adrenal cortex (Bornstein et al., 2000). Furthermore, as will be outlined in section 1.8.4, the role of medullary neuropeptides in regulating corticosteroid production is also becoming well established. An understanding of the development of the gland and the control mechanisms regulating growth and hormone production help to make the nature of this cross-talk more apparent.

1.4 Adrenal Development

The adrenal cortex develops from a thickening of coelomic epithelium - the adrenogonadal primordium (AGP) between the urogenital ridge and the dorsal mesentery which in humans can be identified from 4 weeks post conception (dpc), and in mice 9.5-10dpc (Figure 1.4) (Else & Hammer, G. D., 2005; Hatano et al., 1996). This adrenogonadal primordium is characterised by expression of the transcription factor SF-1. As it grows its cells delaminate and invade the underlying mesenchyme, splitting into the distinct adrenal and gonadal primordia, the former with the higher SF-1 expression (33dpc human, 10.5dpc mouse) (Luo et al., 1994). At 41-44 dpc the human adrenal is expressing CYP17 and becoming vascularised, with two distinct zones detectable by 52 dpc.

The inner fetal zone (FZ) histologically resembles the zona reticularis, while the subcapsular definitive zone (DZ) comes to resemble the zona glomerulosa early in the third trimester. By the ninth week of gestation a mesenchymal capsule has formed around the gland, and by the 14th week a third cortical zone - the transitional zone (TZ) - has developed between FZ and DZ (Sucheston & Cannon, M. S., 1968). By late gestation the DZ and TZ have come to resemble the ZG and ZF respectively (Coulter et al., 1996). In addition to the morphological similarities, the localisation of steroidogenic enzymes and cofactors supports the concept that the DZ develops to form ZG, the TZ is analogous to the ZF, and the ZR emerges from the FZ (Ishimoto & Jaffe, 2011)

The adrenal medulla is derived by invasion of the fetal cortex by cells derived from the neural crest. These sympathogonia migrate in nerves from the sympathetic chain and along blood vessels penetrating the cortex at its cranial aspect from 6 weeks post conception (wpc) (Yamamoto et al., 2004). At least some of these cells retain the potential to become neuronal ganglion cells or chromaffin cells (Ernsberger et al., 2005). The chromaffin cells are scattered within the cortex initially and mostly noradrenergic; it is thought that cortisol production from the transitional zone induces PNMT expression and directs the majority to become adrenergic (Jozan et al., 2007; Wurtman & Axelrod, 1965; Finotto et al., 1999). Islands of medullary tissue coalesce into the adult medulla only in the second year of postnatal life (Wilburn et al., 1986), but as discussed there remain pockets of chromaffin cells scattered throughout the cortex in the adult gland.

The cortex of the fetal adrenal is itself an active endocrine organ - the fetal zone produces large amounts of adrenal androgens which are aromatised by the placenta for oestrogen synthesis (Carr & Simpson, E. R., 1981), 1981], while cortisol production is thought to assist in the maturation of the developing fetus, and theorised to have a role in the induction of parturition (Jaffe et al., 1998). There is transient 3βHSD expression in the FZ in the first trimester, then again in the third trimester in DZ and TZ, with this early expression thought to be part of the mechanism for avoiding virilisation, as the cortisol suppresses ACTH production from the fetal pituitary and excess androgen production at this time (Goto et al., 2006).

The neonatal human adrenal gland is relatively large compared to the adult (3-5g at term), but the FZ rapidly involutes and remodels with attendant fall in adrenal androgen secretion shortly after birth (Jirásek, 1980), and the weight of the gland falls by 50% within two weeks. The DZ and TZ become the ZG and ZF respectively, but it is several years before ZR cells are seen, with a continuous ZR only seen following adrenarche (Belgorosky et al., 2008).

An additional X zone adjacent to the medulla in the mouse appears in the second week of life, and occupies a third of the cortex by the time of weaning (Jones, I. C., 1948). It degenerates at puberty in males or first pregnancy in females, and may derive from fetal zone cells (Morohashi & Zubair, 2011).

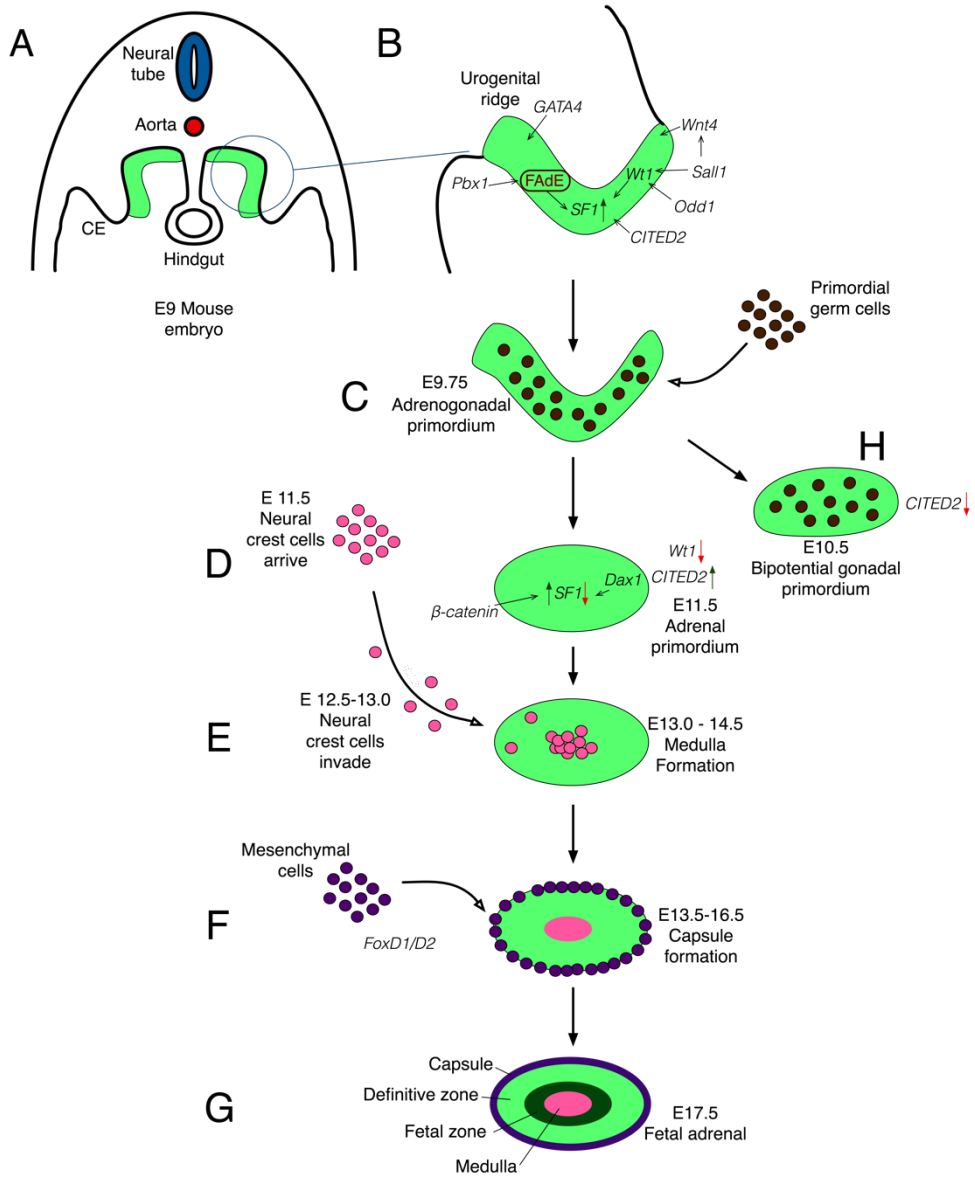

Figure 1.4 Development of the mouse adrenal gland
The adrenogonadal primordium (AGP) develops as a thickening of the coelomic epithelium (CE) adjacent to the hindgut dorsal mesentery [A]. Expression of steroidogenic factor 1 (SF1) is the key step in adrenal development. Wt1 and Cited2 together drive high levels of SF-1 required for AGP development. The more medial cell population with the highest SF-1 expression delaminates into the mesenchyme to form the adrenal primordium (AP)[C], while the cells adjacent to the mesonephros expressing lower levels of SF-1 are invaded by primordial germ cells and delaminate to form the gonadal primordium (GP)[H]. Cited2 is upregulated and Wt1 downregulated in the AP [D]; Cited2 is downregulated in the GP [H]. Migrating neural crest cells arrive at the urogenital region at around day 11.5 [D], entering the AP at approximately day 13 (Val et al., 2007) [D] and subsequently coalescing to form the adrenal medulla by around day 14.5 (Lumb & Schwarz, 2015) [E]. Surrounding mesenchymal cells form a capsule from day 13.5-16.5 (Bandiera et al., 2013), associated with the expression of the transcription factors FoxD1 and FoxD2 (Itäranta et al., 2009; Kume et al., 2000)[E]. The role of an adrenal gland specific enhancer of SF-1 (FAdE) is thought to be key in fetal adrenal development and zonation (Zubair et al., 2006; Zubair et al., 2008). This is active in mice from 10.5dpc when the AGP separates, until 13.5dpc in all AP cells, then from

15.5dpc only in the inner cortex around the medulla, and persisting in the X zone of the adult mouse until its regression. The FAdE contains binding sites for Pbx1 and SF-1 itself (Gardiner et al., 2012). Pbx1 is thought to be required for initiation of SF-1 transcription, with prolonged expression requiring SF-1 to act in a feedforward mechanism. Differential FAdE activation is one proposed mechanism for initial zonation into a fetal zone and a definitive zone (Morohashi & Zubair, 2011). Upregulation of SF-1 by β-catenin and downregulation by Dax1 is described in more detail in the text.

1.4.1 Growth and zonation

The adrenal cortex is a dynamic organ, with the ability to adapt rapidly to changes in the prevailing hormone milieu both in terms of structure and function, and a remarkable regenerative capacity in the face of surgical injury. The centripetal migration hypothesis describes a model whereby there is a continual production of new cortical cells at the periphery of the gland, which then migrate to the centre of the gland before they die at the inner corticomedullary boundary (King, P. et al., 2009). The majority of mitoses in rat and mouse adrenals occur at the ZG/ZF boundary, while most cell death occurs at the ZR/medulla boundary (Rebuffat et al., 1992). Surgical enucleation of the ZF and medulla from the rat adrenal reveals that the adrenal cortex can completely regenerate within 4 weeks, while a similar regeneration also occurs in human adrenals (Baker & Baillif, 1939). Lineage tracing studies following the fate of subcutaneously injected trypan blue revealed that it was taken up initially by capsule and ZG cells, before being observed in the ZF and eventually the ZR (Salmon & Zwemer, 1941). Mosaic transgenic studies provide more contemporary evidence for this theory - columns of β-galactosidase expressing cells under the control of the CYP21 promoter traverse the cortex from capsule to medulla boundary (Iannaccone et al., 2003), and recent genetic lineage tracing experiments have provided direct evidence for centripetal migration in the rat (Huang et al., 2010; King, P. et al., 2009). Chang *et al* demonstrated that centripetal migration also occurs within the mouse adrenal, in a series of 5-bromo-2'-deoxyuridine (BrDU) labelling experiments (Chang et al., 2013). Labelled cells were initially present in the outer cortex, but were detected in the inner cortex after 1 week. In addition, there was evidence for bidirectional movement, with some cells in the outer cortex moving towards the capsule.

Many authors have concluded from these experiments that the progenitor cells come from the capsule, but there is also evidence that they lie within the ZG (Kim et al., 2009). The DZ cells possess characteristics of proliferating rather than steroidogenic cells - expression of proliferating cell nuclear antigen (PCNA) and their relatively lipid-free state (McNutt & Jones, A. L., 1970) - and many studies have identified proliferating cells in the rat

adrenal cortex at the periphery of the gland between the ZG and ZF(Bertholet, 1980). Mitani et al have demonstrated that the majority of the proliferating cells are located around the ZU, and suggest that it is a stem cell zone in the rat adrenal(Mitani et al., 2003).

1.5 Molecular biology of adrenal development

Identification of genes involved in adrenal development has come largely from knock out mouse models and genetic analysis of human adrenal insufficiency.

1.5.1 Urogenital ridge development

The transcription factor Odd skipped related 1 (Odd1) is involved in embryonic patterning in the mouse, with intermediate mesodermal expression at 8.5dpc (So & Danielian, 1999). Odd1 null mice die *in utero* with impaired urogenital ridge formation; those surviving to 15.5dpc show complete absence of adrenals, gonads and kidneys (Wang, Q. et al., 2005).

Germline mutations in Wilms Tumour gene WT1 cause gonadal and renal defects in humans(Baird et al., 1992; Haber & Housman, 1992), and Wt1 null mice die at 13.5 dpc without significant urogenital ridge formation(Kreidberg et al., 1993). A WT1 transgene will allow them to survive to birth but with impaired adrenal development (Moore et al., 1999). Wt1 expression is turned off prior to separation of adrenal and gonadal primordia (Nachtigal et al., 1998; Val et al., 2007).

Sall1 is a transcription factor expressed in murine intermediate mesoderm at 10.5dpc and metanephric mesenchyme at 11.5dpc (Nishinakamura et al., 2001), and in human adrenal FZ (Ma et al., 2002). Sall1 null mice have severe kidney dysgenesis and hypoplastic adrenals at birth, while heterozygous mutations in the gene cause the renal and genital abnormalities of the Townes Brocks syndrome in humans (Kohlhase et al., 1998).

Without the Pbx1 transcription factor mice die at 15-16 dpc with multiple organ aplasia including kidney and adrenal (Selleri et al., 2001; Schnabel et al., 2001). It is expressed

in the urogenital ridge, then adrenogonadal primordium, and throughout the human fetal adrenal cortex by 10 weeks gestation (Ferraz-de-Souza et al., 2009). Heterozygous knockout mice have adrenals with a ZF containing hypertrophied cells in smaller numbers than normal, and fewer proliferative cells in the subcapsular region (Lichtenauer et al., 2007).

The Wnt family of secreted glycoproteins have crucial roles in cell growth and differentiation. Wnt4 is first expressed in the metanephric mesenchyme on the medial side of the ureteric bud (Nishinakamura et al., 2001) and the coelomic epithelium and mesenchyme of the genital ridge at 11.5dpc in mice, but by 14.5dpc it is localised only in the subcapsular region of the adrenal primordium (Heikkilä et al., 2002). A WNT4 homozygous missense mutation in humans is associated with adrenal hypoplasia from 19 weeks gestation (Mandel et al., 2008), while Wnt4 null mice have gonadal defects as well as adrenals with reduced CYP11B2 expression (Heikkilä et al., 2002).

FoxD1/FoxD2 are members of the forkhead/winged helix family of transcription factors. Both are expressed in nephrogenic mesenchyme and in the adrenal capsule (Hatini et al., 1996), and homozygous null mice have hypoplastic adrenals and urological defects at birth (Kume et al., 2000).

1.5.2 Adrenogonadal primordium development

SF-1 is an orphan nuclear receptor transcription factor that is a key regulator of adrenal and gonadal development, and of steroidogenesis itself. It is the earliest marker of the adrenogonadal primordium, with highest levels of expression at the rostral adrenal end (Hatano et al., 1996). SF-1 expression is maintained in all steroidogenic cells of the adrenal cortex when the primordia separate, and it remains high in the steroidogenic cells of the testis, although there is a temporary decline in expression in the ovary in midgestation (Ikeda et al., 2001). Forced expression of SF-1 will differentiate mouse embryonal stem cells and bone marrow cells into steroidogenic cells (Crawford et al., 1997), and expression under the control of its fetal adrenal enhancer leads to the formation of larger adrenals and ectopic adrenal tissue, indicating that non-adrenal lineage cells have switched cell fate (Zubair et al., 2006)

Homozygous deletion of the Sf-1 gene in mice causes loss of the adrenals and gonads by 12.5dpc (Luo et al., 1994; Sadovsky et al., 1995): the coelomic epithelium thickens, but the cells apoptose. Offspring have XY sex reversal and die from neonatal hypoglycaemia. $Sf1^{+/-}$ mice are viable, but have small adrenals which are less responsive to stress (Babu et al., 2002; Bland et al., 2000). Only heterozygous loss of function mutations have been identified in humans, and they much more commonly affect sexual development than adrenal development (Ferraz-de-Souza et al., 2011).

Dax1 (dosage sensitive sex reversal adrenal hypoplasia congenital (AHC) critical region on the X chromosome) is a nuclear receptor transcription factor with an atypical DNA binding domain. It negatively regulates transcription, expressed in the same tissues as SF-1 and acting as its corepressor (Hanley et al., 2001). Mutations in DAX1 in humans cause a form of adrenal hypoplasia congenita (AHC) (Muscatelli et al., 1994; Zanaria et al., 1994) in which patients present with adrenal insufficiency of both glucocorticoids and mineralocorticoids. The adrenal cortex has poorly developed adult zones but retention of cytomegalic FZ cells. Deletion of Dax1 exon 2 has the surprising effect in mice of enhancing steroid output (Yu et al., 1998) until the cortex starts to regress and cytomegalic cells appear as the animals age, suggesting that the mutation is hypermorphic in mice (Scheys et al., 2011). This may be a consequence of Dax1 repression of Oct3/4m, which when activated in embryonal stem cells can cause differentiation (Sun et al., 2009). Loss of Dax1 may cause uncontrolled differentiation of adrenal stem/progenitor cells, with their exhaustion taking longer in mice than humans resulting in a delayed regression of the cortex (Scheys et al., 2011). Levels of SF-1 are also reduced by mutations in the mouse polycomb protein CBX1, which leads to adrenal and gonadal hypoplasia. (Katoh-Fukui et al., 1998).

The insulin-like growth factors also have a role in adrenal development. The IGF1 receptor (IGFR1) is expressed in human fetal FZ and DZ, IGF2 is expressed at high levels throughout the gland, and IGF1 in the capsule only (Shigematsu et al., 1989; Mesiano et al., 1997). A constitutive deletion of the insulin receptor (Insr) and the IGFR1 in a mouse model results in reduced growth, gonadal dysplasia with male to female sex reversal and a range of severe adrenal developmental defects (Pitetti et al., 2013). There are 40% fewer SF-1 positive progenitor cells in the urogenital ridge and a decrease in total Sf-1 transcript levels assessed using a qPCR approach. It is hypothesised that signalling

through these receptors may upregulate SF-1 expression or phosphorylate and thereby enhance its transcriptional activity.

1.5.3 Adrenal primordium development

Cited2 (CBP/p300-interacting transactivator with ED-rich tail 2) is a transcriptional co-factor that binds with high affinity to these eponymous transcriptional co-activators; it can both positively and negatively regulate transcription. Deletion of Cited2 in mice is embryonically lethal (Bamforth et al., 2001) with embryos exhibiting markedly reduced adrenal development by 12 dpc (Val et al., 2007) and adrenal agenesis by 17.5 dpc (Bamforth et al., 2001), as well as cardiac, neural tube and left-right patterning defects (Bamforth et al., 2004).

β-catenin is a protein with two major functions: the majority regulates cytoskeletal binding to cadherin complexes, while a cytoplasmic pool is the defining feature of the canonical Wnt signalling pathway mentioned earlier. Wnt signalling allows nuclear accumulation of β-catenin and thus co-activation of the TCF/LEF family of transcription factors (Wakil & Lalli, 2011). Transcriptionally active β-catenin has been detected in the coelomic epithelium of the adrenogonadal primordium, and later localised to subcapsular cell clusters (Kim et al., 2008). Using an SF-1 cre driver system to delete β-catenin in murine steroidogenic cells only, results in a detectable adrenal primordial at 12.5dpc, but with fewer cells expressing SF-1, and less expression of CYP11A1 and 3βHSD.

As discussed, SF-1 is the major driver of adrenal development and function, and it is through changes in SF-1 expression that many of the genetic defects described are thought to act. Deletion of both Odd1 and Sall1 causes reduced Wt1 expression; Wt1 and Cited 2 together drive the high levels of SF-1 expression required for adrenal primordium development (Val et al., 2007). SF-1 upregulates its own negative regulator, Dax1, perhaps as a mechanism for fine tuning expression during adrenal development, but Dax1 can also function as an SF-1 coactivator at high levels. SF-1 and β-catenin co-operate to transcriptionally activate Dax1 expression (Mizusaki et al., 2003). Wnt4 expression is reduced in Sall1 and Wt1 null mice, and can itself upregulate Dax1 expression.

1.6 Signalling pathways involved in adrenal development

1.6.1 Shh signalling

Sonic hedgehog (Shh), along with Desert hedgehog (Dhh) and Indian hedgehog (Ihh), belongs to the vertebrate hedgehog family of secreted ligands which have a multitude of vital roles during embryonic development and in tissue maintenance, differentiation and regulation of stem cell populations in the adult (King, P. J. et al., 2008). Hh signal receiving cells express the transmembrane protein Patched-1 (Ptch1), which in the absence of ligand binding inhibits the actions of the G-protein coupled receptor Smoothened (Smo), and ensures that the downstream Zn-finger transcription factors Gli2 and Gli3 are subjected to an inhibitory proteolytic processing (Pan et al., 2006). Hh binding to Ptch 1 allows Smo to prevent this processing, so Gli2 and Gli3 can operate as transcriptional activators. The pathway also upregulates Gli1, which is a useful marker for active Hh signalling (Vokes et al., 2007).

Sonic hedgehog is expressed in the adrenal primordium in subcapsular cells, and remains restricted to this population throughout gestation and adulthood (Ching & Vilain, 2009; King, P. et al., 2009; Huang et al., 2010). Shh expression in rodents occurs in relatively undifferentiated but steroidogenic cells expressing neither CYP11B1 or B2, arranged in a continuous layer between ZF and inner ZU in rats(Guasti, Paul, Laufer & King, 2011a), while in humans and mice they are arranged in clusters between cells of the ZG (King, P. et al., 2009).

Defects in adrenal development have been seen as a results of inactivation of the Hh pathway. Holoprosencephaly is often accompanied by adrenal hypoplasia with defects in SHH, PTCH1 and GLI2 genes (Nanni et al., 1999; Ming et al., 2002; Roessler et al., 2003), while adrenal agenesis is seen in Pallister Hall syndrome, the result of a GLI3 mutation. Homozygous deletion in mice is embryonically lethal, but prior to this the adrenal primordial is noted to be much smaller than wild type (Ching & Vilain, 2009). Deletion from only steroidogenic tissue results in animals whose adrenals are small, but with intact zonation and a normal sized medulla (King, P. et al., 2009).

Shh expression has been detected in the developing mouse adrenal almost immediately after its separation from the AFP (King, P. et al., 2009). Analysis of Shh expression using

a Shh cre driver shows expression as early as 11.5dpc, increasing to 13% of all SF-1 positive cells by 12.5dpc but mainly subcapsular, then to 75% of SF-1 positive cells by 13.5dpc, but spread throughout the cortex. All the cortical cells are labelled in these adrenal glands when harvested after birth. When similar experiments are performed with an inducible Shh cre-T2 driver, labelled cells were seen at the periphery of the gland following tamoxifen injection at 14.5dpc, and after 28 days' columns of cells were observed spanning the cortex from capsule to medulla. Similar results were obtained in the adult after 7 or 13 days, with labelled cells co-expressing CYP11B1 or CYP11B2. These data identify the Shh-expressing cells as candidate stem/progenitor cells.

The cell population that are responding to the secreted Shh signal are mesenchymal cells in the capsule and periphery of the cortex. They do not express SF-1 or CYP11A1 but do express Gli1, Ptch1 and FoxD2. Deletion of Smo from cortical cells using the SF-1 cre driver does not affect adrenal development, but the adrenal hypoplasia associated with Shh deletion is associated with a much thinner capsule whose proliferative capacity is greatly reduced (King, P. et al., 2009). This suggests that the effect of Shh deletion is mediated via the capsule. Using inducible Gli1 cre drivers in lineage tracing experiments demonstrates that following tamoxifen injection at 14.5dpc there is initial labelling of the Shh-responsive cells in capsule and subcapsular mesenchyme (SF-1 negative), but that clusters of labelled cells which do express steroidogenic markers are observed in the cortex and expand into columns with extended chases (King, P. et al., 2009; Huang et al., 2010). Interestingly, a proportion of these cells are observed to express Shh itself. Similar columns of cells are seen following postnatal induction of the Gli1 cre driver.

Taken in concert, these lineage-tracing studies provide a direct confirmation for the centripetal migration hypothesis, and the periphery of the gland as the location for the stem/progenitor population. The implication is that Shh signals to a mesenchymal population of cells that differentiate from a non-steroidogenic to a steroidogenic phenotype, at least in part via a Shh-expressing intermediate. The definitive cortex develops from the capsule, and is of both coelomic epithelium and mesenchymal origin. Wood and Hammer (Wood & Hammer, G. D., 2011) have proposed a model wherein cells in the newly separate adrenal primordium enter the developing adrenal capsule and have their SF-1 expression turned off before Shh expression in the cortex begins; SF-1 expression is reactivated upon entry of these cells back into the cortex. The transcription

factor Pod1 is a candidate for the factor which represses SF-1: loss of Pod1 in mice leads to increased differentiation of steroidogenic Leydig cells in the testis, and ectopic expression of the SF-1 and SF-1 target genes (Cui et al., 2004). Adrenal expression in the adult mouse is exclusively in the capsule, where Pod1 null mice also show ectopic SF-1 expression (Kim & Hammer, G. D., 2007). However, other data are not in support of this hypothesis – an SF-1 BAC transgene does not label the capsule, and Pod1 expression is not detected in the capsule at 16.5dpc in the mouse when SF-1 is undetectable (King, P. et al., 2009).

There is a reduction in PCNA staining in Shh mutant adrenals compared to wild type, but no apparent increase in apoptosis, suggesting that Shh signalling to the capsule is generating a proliferative rather than a survival signal to the cortex (Ching & Vilain, 2009). Huang suggests that Shh acts as a mitogen for the capsule, hence the thin capsule is Shh mutant adrenals, but an alternative explanation is that Shh acts as a chemoattractant to promote the coalescence of surrounding mesenchyme to form the capsule itself. A further hypothesis has it that Shh regulates the differentiation of the capsule into the steroidogenic lineages, and the capsule depletes rapidly of these cells when it is absent. The precise nature of how the signal from capsule to cortex is transduced has yet to be determined, but possible candidates include the Wnt signalling pathway, and fibroblast growth factors.

1.6.2 FGF signalling

Fibroblast Growth Factor (FGF) signalling controls a number of early developmental processes: cell movement during gastrulation, mesoderm and neuroectoderm formation, anterior/posterior patterning and organogenesis (Ornitz & Itoh, 2001). 22 members of the FGF family have been identified, all characterised by a conserved core of 140 amino acids, and all except for the intracellular FGFs (iFGF, FGF11-14) signal through the FGFRs. These are receptor tyrosine kinases, binding to which most commonly activates Ras/MAPK pathways to control cell proliferation and differentiation, Akt to determine cell survival or protein kinase C to regulate cell migration (Dorey & Amaya, 2010; Bottcher & Niehrs, 2005).

FGFs 2 and 9 are expressed predominantly in the adrenal capsule, and FGFRs 2 IIIb, 2 IIIc and 3 IIIc are expressed mainly in the cortex, with FGFR 2 IIIb and IIIc expression in subcapsular clusters of cells similar to Shh and β-catenin (Guasti et al., 2013). FGF2 is a mitogen for adrenocortical cells in culture and gland regeneration experiments (Lepique et al., 2004) and binds specifically to ZG cells, where FGFR2 IIIc is located. Deletion of both isoforms of FGFR2 leads to hypoplastic adrenals and male to female sex reversal, implying that FGFR2 is required for adrenal growth and development after AGP formation. FGFR2 IIIb null mice have thickened capsules with disorganised cells, with enhanced cell proliferation and upregulated Gli1 expression, suggesting that FGF signalling may actually be a negative regulator of Shh expression (Chida et al., 2007).

1.6.3 POMC and ACTH

ACTH is a product of the proteolytic cleavage of the prohormone pro-opiomelanocortin (POMC) in the pituitary gland and a variety of extra-pituitary tissues (Dores & Baron, 2011). Pituitary POMC is expressed in anterior lobe corticotroph cells and intermediate lobe melanotroph cells, although the intermediate lobe is not prominent in humans.

POMC gene:

The POMC gene is 8-kb and located on chromosome 2p23 in the human, and consists of three exons and two intervening introns (Fig 1.5a). The first exon encodes a leader sequence, the second encodes the signal initiation sequence and the N-terminal portion of POMC, and the third encodes most of the mature peptide sequences. Transcription is initiated in the pituitary from a promoter which generates an mRNA transcript of approximately 1200nt. In extra-pituitary tissues a downstream promoter is capable of generating truncated 800nt transcripts arising from the 5' end of exon 3, and longer 1380nt transcripts via an upstream promoter. This was demonstrated by Clark *et al* using Northern Blot analysis of tissue from ACTH-secreting pancreatic neuroendocrine tumours (Clark et al., 1989). In human placental tissues all three transcripts are present: a pituitary-like 1200nt transcript in addition to the 800nt and 1380nt species (Grigorakis et al., 2000). Translation of the shorter peptides can potentially be initiated from the methionines (ATG) indicated in figure 1.5a, but they lack the signal sequence for targeting to the ER, and it is felt unlikely that these peptides would be secreted (Jeannotte et al., 1987).

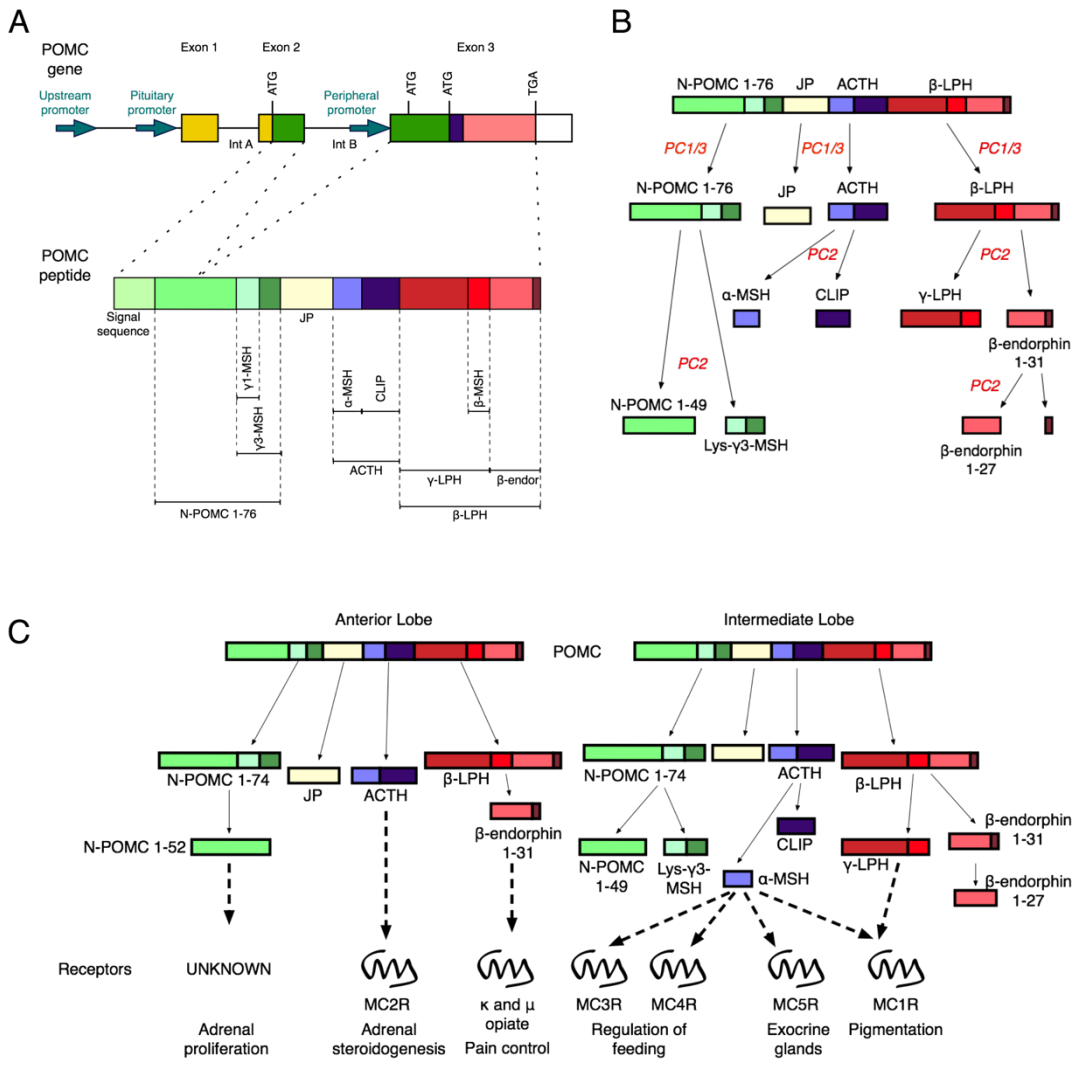

Figure 1.5 POMC processing

A: The POMC gene consists of three exons and two introns. A pituitary selective promoter produces a transcript of 1200nt, in extra-pituitary tissues a downstream promoter can produce 800nt transcripts (downstream promoter) and 1380nt transcripts (upstream promoter). The 800nt transcripts can potentially be translated from an ATG site just upstream of ACTH, but these lack the signal sequence for targeting to the secretory apparatus.

B: The POMC proprotein convertase processing enzymes PC1/3 and PC2 are differentially expressed in the` anterior and intermediate lobes of the pituitary. PC1/3 expression alone will generate ACTH, but high levels of PC2 in the intermediate lobe lead to the production of alpha-MSH

C: POMC peptides act mainly through a family of five G-protein coupled receptors known as the melanocortin receptors (MCX-R where X is a number from 1–5). β-endorphin acts via the κ and μ opiate receptors and the receptor through which the N-terminal peptides stimulate adrenal mitogenesis remains to be identified. ACTH, adrenocorticotrophin; CLIP, corticotrophin-like intermediate peptide; LPH, lipotrophin; MSH, melanocyte-stimulating hormone. Adapted from (Bicknell, AB, 2008). Anterior lobe processing as shown in the figure occurs in humans as well as other animals. The presence of any significant intermediate lobe in humans is disputed, as the fetal

pars intermedia regresses soon after birth although immunoreactive alpha-MSH is seen in the zona intermedia of the human gland, between anterior and posterior pituitary (Coates et al., 1986). Both anterior and intermediate lobe-like POMC processing takes place in the central nervous system (Bicknell, AB, 2008) and in neuro-endocrine tumours (Beuschlein & Hammer, G. D., 2002).

Control of POMC expression:

In the pituitary gland expression of POMC is under multihormonal control with the main stimulatory input from CRH and AVP, and inhibitory regulation from adrenal glucocorticoids(Drouin, 2016). The role of neuropeptides and cytokines in stimulating steroid production in the adrenal will be described later, but there is a similar level of involvement in control of POMC gene expression and production of POMC peptides, with factors such as serotonin, oxytocin and leptin, along with pro-inflammatory cytokines, all involved (Slominski et al., 2000).

The role of methylation status of the POMC promoter in enabling POMC expression has been established by Newell Price *et al* (Newell-Price et al., 2001). They demonstrated that the POMC promoter is embedded within a defined CpG island, much of which is methylated in non-expressing tissues, and specifically un-methylated in POMC-expressing tissues and tumours. Ye *et al* (Ye et al., 2005)defined a region between -417 and -260 bp upstream of the POMC transcription initiation site where the levels of POMC expression correlated inversely with methylation density.

There are a variety of transcription factors whose restricted pituitary expression leads to cell-specific POMC expression. The key attributes are an enhancer region with a site that cooperatively binds TF pituitary homeobox 1 (Pitx1) and Tbox TF Tpit (Tbx19), and a further binding site which is a target for neurogenic differentiation 1 (NeuroD1) (Poulin et al., 2000) – together these two regulatory elements synergistically control transcription of the *POMC* gene in corticotrophs. Melanotroph-specific gene expression is enabled by the expression of the transcription factor Pax7 (Budry et al., 2012): in Pax7-/- mice expression of melanotroph markers such as PC2 (see below) in the intermediate lobe are severely reduced, while corticotroph markers such as the CRH and vasopressin V1b receptors are upregulated.

POMC Processing:

POMC processing is shown in figure 1.5b and c. Proprotein convertases are widely expressed in endocrine tissues and catalyse the cleavage of prohormones including proPTH and proinsulin (Chrétien, 2011). The serine proteases PC1/3 and PC2 are the members of this family of enzymes involved in the processing of POMC. PC1/3 is predominant in the anterior lobe, while in the intermediate lobe both PC1/3 and PC2 are expressed (Zhou et al., 1993). This is the molecular basis for the generation of predominantly ACTH from the anterior pituitary, while smaller POMC peptides such as α-MSH are produced in the intermediate lobe.

The presence of an intermediate lobe in humans is debated, as the fetal pars intermedia regresses soon after birth, and there is no distinct intermediate lobe on histological examination (Horvath, E. et al., 1999). There is no pituitary secretion of alpha-MSH in normal physiological circumstances, supporting the idea that the intermediate lobe is not a significant presence (Iino, K. et al., 2010). It is reported, however, that immunoreactive alpha-MSH is seen in the zona intermedia of the human gland, between anterior and posterior pituitary (Coates et al., 1986); and that there is an. increase in circulating levels of α-MSH in the serum of Addison's disease patients (Donahoo et al., 2009). PC2 expression is reported in both normal human pituitary (Hook et al., 2009; Lloyd et al., 1995) and pituitary adenomas (Jin et al., 1999; Iino, K. et al., 2010); and pituitary dependent Cushing's disease is associated with the presence of smaller POMC peptides such as α-MSH (Colao et al., 1993; Coates et al., 1986). Levels of PC2 expression in tumours associated with ectopic production of ACTH are significantly higher than in ACTH-secreting pituitary adenomas (Tani et al., 2011). In conclusion, the literature to this point suggests that hormone production from the pituitary intermediate lobe does not play a physiological role in humans, although intermediate lobe-like POMC processing does occur in endocrine tumours from the pituitary and elsewhere.

ACTH in fetal adrenal development:

Much of the early evidence for the role of fetal pituitary ACTH in adrenal development comes from the study of anencephalic fetuses. In this common congenital abnormality of varying severity a major portion of the brain, skull and scalp is absent – generally fetuses lack a hypothalamus but often the anterior pituitary is retained (Swaab et al., 1978). Early adrenal development proceeds normally, suggesting that hypothalamic input at least is not required for this, but there is often adrenal hypoplasia between 13-18 weeks post

conception (Benirschke, 1956; Mazzitelli et al., 2002). Microscopic examination reveals a structurally intact DZ and medulla, but a small FZ with diminished DHEA production. Pituitary ACTH is detectable at around 50dpc, contemporaneous with adrenal steroidogenic enzyme expression, and thought to be responsible for FZ androgen production (Goto et al., 2006). Pituitary glands in anencephalic fetuses have decreased ACTH production by midgestation, so there is an ongoing requirement for hypothalamic stimulation; ACTH administration to anencephalic fetuses in utero is able to restore the size of the FZ.

Based on these data it has been proposed that ACTH, or possibly another POMC peptide (Estivariz et al., 1988), is the primary regulator of adrenal development. However, Pomc null mice (Karpac et al., 2005) and Mc2r null mice (Chida et al., 2007) have normal adrenals at birth, indicating that neither ACTH, nor any other POMC peptide, is required for fetal adrenal development in this species. It is possible that because the rodent placenta, unlike humans, expresses CYP17 and is able to make oestrogen (Arensburg et al., 1999; Durkee et al., 1992), whereas in humans this is formed from adrenal androgens secreted from the FZ under the control of ACTH, there is no need for ACTH signalling in utero in mice. This would indicate that the role of ACTH in humans is to maintain pregnancy through its actions on steroidogenesis rather than via its effects on growth. ACTH is not mitogenic for adrenocortical cells grown in vitro, and in some systems has shown to be anti-mitogenic (Masui & Garren, 1971; Rocha et al., 2003). It has been hypothesised that ACTH upregulates the expression of other growth factors locally, such as IGF2 (Voutilainen & Miller, 1987) and FGF2 (Mesiano et al., 1991), both of which themselves are potent mitogens for adrenocortical cells in vitro (Crickard et al., 1981). IGF2 is not expressed in the adult cortex, but is proposed to be a major regulator of fetal adrenal growth (Mesiano et al., 1997).

1.7 Postnatal growth, zonation and remodelling

The postnatal and adult adrenal cortex is highly dynamic: in addition to the continuous centripetal migration of cells described under normal conditions, the relative sizes of the cortical zones can be remodelled in response to hormonal requirements, and the regenerative capacity of the gland following injury is considerable.

1.7.1 Zonation

The mechanism by which the functional zonation of the adrenal cortex is defined has been described above, but the manner in which this is established and thereafter altered in response to the organism's hormonal requirement is becoming increasingly understood. The centripetal migration theory would predict the ability of ZG cells in the outer cortex of the mouse adrenal to transdifferentiate into their adjacent ZF cells, but an intervening undifferentiated zone (ZU) in rats presents a conundrum for the maintenance of zonation in this species. The ZU is further subdivided into an outer Shh-expressing oZU and smaller inner layer that does not express Shh (iZU)(Guasti, Paul, Laufer & King, 2011b). The juxtaposition of the proposed capsular or subcapsular stem/progenitor cells with the ZG and ZF raises some interesting questions regarding the differentiation processes that occur during centripetal migration.

Capsular cells delaminating and entering the cortex would seemingly first become CYP11B2-positive ZG cells, not Shh-positive cells. Mitani et al respond to this with the idea that the capsular cells instead migrate through the CYP11B2-expressing ZG layer of cells to the oZU, and from there can differentiate in either the ZF or ZG direction (Mitani et al., 2003). Interestingly, they observed a circadian rhythm to this process, with a peak at 4am for differentiation into ZF, and at 8pm for differentiation into ZG cells. This is in keeping with Miyamoto et al (Miyamoto et al., 1999), who showed a burst of cell proliferation in the outer ZF at 3-4am. Such bidirectional movement from the ZG/ZF boundary is also observed in the mouse adrenal by Chang et al (Chang et al., 2013). The iZU has very high levels of steroidogenic enzyme expression, and expresses the MC2R and its accessory protein MRAP (Gorrigan et al., 2011), perhaps because this zone is a transitional one, where Shh expression has been lost again, but the definitive identity of CYP11B1 or B2 positivity has not yet been achieved. A pool of such CYP11B1 and B2 negative cells has been found in the human adrenal cortex, hinting that the rat ZU does have a human equivalent.

Similarly in ZF, chronic ACTH administration expands this zone with increased CYP11B1 expression, but HPA suppression from exogenous dexamethasone contracts the zone (Dallman, 1984). The mechanisms by which these expansions and contractions occur may involve induced differentiation from stem/progenitor cell populations or trans-differentiation from another zone, possibly via a de-differentiated Shh-expressing cell

type (McEwan et al., 1996). McEwan *et al* have observed enhanced ZU proliferation when the HPA or RAS axes are chronically activated (McEwan et al., 1996), and Mitani *et al* have reported migration of BrdU labelled cells from ZU to ZG when the RAAS is activated (Mitani et al., 2003).

Freedman *et al* performed cell fate mapping in the adrenals of aldosterone synthase (AS)/Cre x $R26R^{mT/mG}$ reporter mice, in which Cre-mediated activation of a cell lineage marker in differentiated ZG cells is driven by the AS gene promoter (Freedman et al., 2013). They reported trans-differentiation of lineage-marked ZG cells into ZF cells in the first twelve weeks of life, and also in the recovery period following HPA axis suppression with dexamethasone. However, lineage conversion did not occur in mice in which SF-1 had been deleted specifically in the ZG. It was also reported that under alternative stress conditions, i.e. in ZG-specific SF1 knockout mice which retain a functional ZF, differentiated fasciculata cells could derive from non-Cyp11b2-expressing cells, suggesting that the must be other means of deriving differentiated ZF cells. This is supported by the possibility of bidirectional migration of cells into the ZG and ZF cell from a putative progenitor population located at the ZG/ZF interface mentioned earlier.

The paradigm of adrenal regeneration following surgical injury seen when the cortex is regenerated from just remaining capsule and subcapsular adherent ZG cells, or when the contralateral gland undergoes hypertrophy following unilateral adrenalectomy provides further insights. In the rat adrenal following enucleation, the remaining ZG cells lose CYP11B2 expression before cortical proliferation replaces the lost cells, while there is an initial thickening of the capsule, indicating potential involvement of capsule cells in regeneration (Reiter & Pizzarello, 1966). Following unilateral adrenalectomy, cell proliferation in the outer ZF is seen within 2 days, although there is no increase in ZG proliferation. Interestingly, no such compensatory growth is seen in $SF\text{-}1^{+/-}$ mice (Beuschlein et al., 2002). This latter observation supports the idea from Freedman *et al* on the importance of SF-1 expression in ZG to ZF trans-differentiation.

The expression of MC2R and MRAP in the iZU would suggest a role in causing differentiation to the ZF phenotype, possibly via a downregulation of Dax1 expression. The role for Dax1 in inhibiting steroidogenesis has been discussed, but it is also a negative regulator of embryonic stem cell differentiation, and therefore may be a modulator of

adrenal stem cell mobilisation. Inhibition of the HPA has not been consistently shown to inhibit compensatory growth after unilateral adrenalectomy however: dexamethasone inhibits the process in some studies (Engeland et al., 2005; Krueger & Orme-Johnson, 1983; Phillips et al., 1985) but not others (Grizzle & Dunlap, 1984), while hypophysectomy blocks regeneration following enucleation(Estivariz et al., 1988) but does not completely block compensatory growth (Dallman, 1984). Hypophysectomy does reduce the extent of compensatory growth, however, but the precise identity of the pituitary factor involved is unclear.

1.8 Control of adrenal cortex proliferation and steroidogenesis

As mentioned, adrenal steroid output adjusts rapidly in response to acute changes in HPA or RAS activity, and chronic activation is accompanied by profound changes in the size and steroidogenic gene expression of the appropriate zone.

1.8.1 Zona Glomerulosa and the RAS

Rodent studies have confirmed how prevailing low sodium or high potassium conditions expands the ZG to generate more CYP11B2 expressing cells, and therefore more aldosterone, to correct the physiological imbalance (Romero, Yanes et al., 2007). RAS suppression with a high sodium diet or administration of angiotensin converting enzyme (ACE) inhibitors, causes the ZG to shrink rapidly with a reduction in both CYP11B2 expression and blood flow (Brennan et al., 2008; Dallman, 1984).

1.8.2 Zona Fasciculata and the HPA axis

The effect of ACTH in inducing ZF glucocorticoid production is established, but its role in regulating proliferation during remodelling is much more controversial. The circadian rhythm of zona fasciculata proliferation is closely matched to that of ACTH (Mitani et al., 2003), while Cushing's disease (excess pituitary ACTH) is associated with adrenocortical hyperplasia (Bertagna et al., 2009). Hypophysectomy of experimental animals leads to adrenocortical hypoplasia, and the Pomc null mice described previously have glands which atrophy soon after birth, in a manner that is correctable by the administration of ACTH (Coll et al., 2004). Such ACTH treatment does not restore the adrenals of the hypophysectomised animals, however (Payet & Lehoux, 1980). Recently the importance of intra-adrenal production of ACTH in the development of ACTH-independent

macronodular adrenal hyperplasia has been proposed. Lefebvre *et al* have demonstrated expression of POMC and bioactive ACTH (1-39) in adrenal nodules in this condition, but without expression of pituitary-specific transcription factors such as Pit1 and Pitx1 (Lefebvre et al., 2011). The authors did not identify which POMC promoter was responsible for transcription in this case – previous observations on extra-pituitary tissues such as adrenal and testis suggest that the major mRNA product is the 800nt transcript, produced via the downstream promoter (Lacaze-Masmonteil et al., 1987). As described previously, the translated product would lack the hydrophilic signal peptide, precluding adequate processing and secretion of ACTH.

The role of POMC-derived peptides other than ACTH in the control of adrenal growth is becoming increasingly well understood. For instance, the N-terminal fragment of POMC, alternatively known as N-POMC or pro-γ-MSH is a 76 residue glycopeptide that is co-secreted with ACTH from the pituitary into the circulation. While antibodies directed at this peptide can block compensatory growth following adrenalectomy, the peptide itself has no mitogenic activity to adrenocortical cells in vitro, although N-POMC1-28 and N-POMC2-54 are potent mitogens in this context (Lowry et al., 1983). The latter are not products of normal pituitary processing, but an enzyme expressed in the outer rat ZF has been identified that can cleave pro-γ-MSH to produce smaller mitogenic peptides. Expression of this adrenal secretory protease (AsP) is increased during compensatory growth (Bicknell et al., 2001). Synthetic N-POMC 1-28 has subsequently been shown to stimulate proliferation of human H295R cells, and to activate the ERK signalling pathway (Fassnacht et al., 2003). Interestingly, N-POMC 1-28 cannot restore adrenal growth in Pomc null mice (Coll et al., 2006).

These studies may suggest that ACTH is not involved in adrenal cell proliferation during adrenal remodelling, but it has been reported that the elevated ACTH levels that accompany enucleation are required for the de-differentiation of the ZG cells that is an immediate response in the regenerating adrenal (Engeland & Levay-Young, 1999). Whether this works via inhibiting Dax1 expression has not been reported. ACTH does not affect proliferation in the ZG; the cell proliferation observed by placing animals on a low sodium diet is inhibited by type 1 angiotensin receptor antagonists (McEwan et al., 1996) indicating that cell growth in this zone is controlled by the RAS (Chatelain et al., 2003). Chang et al identified a population of cells in the outer mouse cortex which did not

express CYP11B1 or CYP11B2, but which were induced to proliferate following acute treatment with ACTH (Chang et al., 2013). This is consistent with the idea that the ZG contains a population of slow-cycling stem or progenitor cells which are activated in response to physiological stimuli.

1.8.3 Autonomic nerve supply

The anatomy of the considerable autonomic nerve supply to the adrenal gland has been described previously. Acetylcholine release from preganglionic sympathetic fibres stimulates release of catecholamines from the medulla, but there is significant evidence for a role in the regulation of glucocorticoid secretion.

Sympathetic innervation of the gland is required to maintain the circadian rhythm of glucocorticoid secretion (Ottenweller & Meier, 1982; Dijkstra et al., 1996; Muglia et al., 1997); and can modulate secretion even in hypophysectomised animals (Engeland & Gann, 1989). Such stimulation also increases adrenal blood flow in dogs and calves, likely mediated by various neuropeptides, which will be described in more detail below (Breslow et al., 1987). There is evidence for a local VIPergic neuron system in the rat adrenal(Hokfelt et al., 1981), and increased VIP staining in the medulla when the splanchnic nerve is ligated(Holzwarth, 1984)

Engeland et al suggested that the phenomenon of compensatory growth following unilateral adrenalectomy was neurally mediated (Engeland et al., 1975). Compensatory growth is blocked by interruption of the nerve supply (Kleitman & Holzwarth, 1985), while mechanical perturbation of the nerves of one adrenal causes increased proliferation in the other (Dallman et al., 1976). This suggests that both afferent and efferent nerves are involved in mediating this process via the hypothalamus. Sectioning of the splanchnic nerve has also been shown to decrease the adrenal response to ACTH (Edwards & Jones, C. T., 1987). Additionally, there is also evidence for electrical coupling between cells of the adrenal cortex via gap junctions, enabling signals received in a few cells to spread more widely through the gland (Usadel et al., 1993; Murray et al., 2009)

1.8.4 Adrenal Neuropeptides

In addition to catecholamine secreting cells, the adrenal medulla also contains multiple population of cells containing a variety of neuropeptides (Siegel et al., 1985; Linnoila et

al., 1980; Dumont et al., 1983). There is also neuropeptide secretion from splanchnic nerves and local intra-adrenal nerve endings, leading to autocrine, paracrine and endocrine effects. The main steroidogenic effect is on the generation of aldosterone, which is stimulated by PACAP, VIP, Substance P, adrenomedullin and Neuropeptide Y (NPY) (Neri et al., 1996; Hinson et al., 1992; Mazzocchi et al., 1995; Mazzocchi et al., 1996; Bernet et al., 1994) as well as ANP and somatostatin synthesised in chromaffin cells. Three of the most well-described neuropeptides are adrenomedullin, PACAP and VIP.

Adrenomedullin (ADM) is a 52 amino acid peptide with homology to calcitonin gene-related peptide that was initially isolated from a human phaeochromocytoma (Bełtowski & Jamroz, 2004). In vitro it inhibits basal and CRH-mediated ACTH secretion from the pituitary, but activates CRH-producing neurons in vivo. As well as expected expression in the adrenal medulla, both ADM and its receptors are expressed in the ZG (Andreis et al., 1997), where it inhibits Ang II-induced aldosterone production, while also increasing basal and ACTH-stimulated aldosterone production (Kapas et al., 1998). There are very few ADM receptors in the ZF and ZR and no discernible effect on cortisol secretion (Ziolkowska et al., 2003). The systemic effects of adrenomedullin are in keeping with a role as a negative regulator of aldosterone action, causing vasodilation, diuresis, natriuresis and having an inhibitory effect on cardiac remodelling (Kato & Kitamura, 2015).

PACAP (pituitary adenylate cyclase-activating peptide) is the most conserved member of the nine hormone superfamily which also includes glucagon, GLP-1, secretin and VIP (Sherwood et al., 2000). There is extensive innervation of the adrenal medulla with PACAP-ergic fibres, where it increases catecholamine secretion by up regulating tyrosine hydroxylase, PNMT and dopamine β-hydroxylase, but it also stimulates aldosterone secretion from the ZG (Bodart et al., 1997; Neri et al., 1996)

Vasoactive intestinal peptide (VIP) content of the rat adrenal is stimulated by a low sodium diet, and it has a synergistic effect on aldosterone secretion from the ZG (Hinson et al., 1992). This is mediated in part at least by VIP-ergic nerves with their origins in the medulla (Holzwarth, 1984) . VIP stimulates catecholamine secretion from the medulla in a similar manner to PACAP (Harmar et al., 2012)

1.8.5 Intra-adrenal immune cells and cytokines

The importance of the immune system in stimulating the adrenal gland via activation of the hypothalamus and pituitary is well established(Jones, T. H. & Kennedy, 1993; Chrousos, 1995), but there is evidence for local immune regulation within the gland itself. There is a population of macrophages found primarily in the ZR, (Gonzalez-Hernandez et al., 1994), but there is also cytokine production from adrenal cells themselves (Bornstein et al., 2004). Mast cells are present in the adrenal capsule, and degranulate releasing 5-HT and histamine when activated by ACTH. Inhibition of mast cell degranulation abolishes the effect of ACTH to increase adrenal blood flow (Stark et al., 1965; Hinson et al., 1991). Chromaffin cells may also produce cytokines (Bornstein et al., 1996), but are more notable for their extensive expression of cytokine receptors: receptors for TNF-α, IFN-α, IL-1 α/β and IL-6 are all present on chromaffin cells (Bunn et al., 2012)

IL-1 can induce glucocorticoid secretion independent of ACTH (Kapcala et al., 1996) in a manner that may be related to local prostaglandin release (Winter et al., 1990). Indeed, IL-1 secretion from an inflammatory mass has been responsible for a single reported case of ACTH-independent Cushing's syndrome. IL-6 also increases cortisol levels independent of ACTH, but its effect is most marked on the production of adrenal androgens. IL-6 is expressed in the ZR and stimulates DHEA secretion (Parker, L. N. & Odell, 1980).(Path et al., 1997), and in humans has a role in maintaining upregulation of adrenal steroidogenesis during chronic stress, independent of the CRH-ACTH axis (Chrousos, 1995; Naito et al., 1992). TNF-α on the other hand, while an inducer of pituitary ACTH secretion, inhibits the local ACTH-induced cortisol production within the adrenal (Voutilainen, 1998).

1.9 Summary

There has been a significant increase in our understanding of the mechanisms controlling adrenal development and function, but with much remaining to be discovered. Definitive identification of the adrenocortical stem/progenitor cells is awaited - do they reside in the capsule, the subcapsular cortex or both? They are likely to have extremely important roles within a gland that is constantly being remodelled according to the requirements of

the organism. The dogma of ACTH stimulating cortisol release has been shown to be a gross oversimplification of a process with multiple different neurohumoral and immune inputs, and further characterisation of these is an exciting prospect.

1.10 Aims

This thesis aims to provide an improved understanding of the mechanisms involved in remodelling of the adrenal cortex, and of the role of POMC derived peptides and melanocortin receptors in stimulating adrenocortical growth and activity.

1. Dietary and pharmaceutical modification of the renin-angiotensin-aldosterone system will be employed to remodel the rat adrenal gland and investigate (i) gross histological changes; (ii) changes in proliferation; (iii) changes in expression of markers of the cell cycle.
2. A case of ACTH-independent macronodular adrenal hyperplasia and Cushing's syndrome will be described. Potential mechanisms for the development of the disease will be explored, with a potential role for a co-existing gastro-intestinal stromal tumour being considered.
3. A variety of histological and immunohistochemical techniques will be employed to (i) Elucidate the mechanism of pigmentation in pigmented adrenal diseases; (ii) understand the role of melanin in normal adrenal physiology.

Chapter 2 Materials and Methods

2.1 Cell culture

Human adrenocortical carcinoma cell line NCI-H295R

H295R cells were grown at 37ºC with 5% CO_2 in a standard media of 50% DMEM(Dulbecco's Modified Eagles Medium), 50% F12(Nutrient mixture F12 Ham) (1:1) supplemented with 100 U/mL penicillin, 100 µg/ml streptomycin (Sigma), 10% fetal bovine serum (FBS; Invitrogen) and 1x insulin-transferrin-selenium solution (10 µg/ml insulin, 5.5 µg/ml human transferrin, and 5 ng/ml sodium selenite; BD Biosciences) (Wang, T. et al., 2012).

It is more common in the published literature to culture H295R cells in a medium that contains a serum substitute rather than fetal bovine serum, for example Nu-serum (Collaborative Biomedical Products, Bedford, MA) or Ultroser-G (2.5%, Pall Corporation, Port Washington, NY) (Tao Wang, 2012). The effects of culture supernatant from another cell line on cortisol production were being assessed (GIST primary culture), so H295R cells were cultured in the same standard medium to provide a valid negative control.

Mouse Melanoma Cell Line B16F10

These cells were kindly provided by Professor John Marshall, Barts Cancer Institute. B16F10 cells were grown at 37ºC with 5% CO_2 in DMEM supplemented with 10% FBS (Invitrogen).

Primary culture

Primary cultures of human GIST and adrenal gland and were obtained as follows. Tissue samples were collected in ice-cold phosphate buffered saline, and treated with type 1 collagenase (Sigma) 1mg/ml and DNase A (Sigma) 10µg/ml in serum free standard media for 2-4 hours at 37ºC with frequent agitation. Cells were collected by centrifugation at 800g, resuspended in 10ml serum free media and strained through a 40 µl cell strainer (BD Biosciences). The strainer was washed twice with 5ml serum free media and cells in the flow through were pelleted by centrifugation at 500g. After a 3 minute treatment with erythrocyte lysis buffer (NH_4Cl 150mM, $KHCO_3$ 10mM, disodium EDTA 10mM) at room temperature the reaction was stopped with ice-cold PBS. Cells were collected by

centrifugation at 500g and plated in standard media containing 10% fetal bovine serum as described for H295R (Mukaisho et al., 2006). All cells were incubated in a humid atmosphere of 5% CO_2 at 37ºC.

Cultured GIST cells had media changed on reaching 80% confluency, then media removed after 48 hours, sterile filtered and stored at -80ºC prior to cell stimulation.

Primary cultures of rat adrenal gland were obtained as follows. Both adrenals were harvested from 4 male Wistar rats, 6 weeks old and approximately 380g each. The surrounding fat was trimmed and the glands were squeezed between two autoclaved microscope slides. The adrenal capsules were removed and the cortex/medulla from all rats was combined and digested according to the protocol described above. Primary cultures of human adrenal cells were

For stimulation of rat adrenal primary cultures cells were initially plated in 12 well plates at a density of $2x10^5$ cells per well and stimulated after 5 days (Ramachandran & Suyama, 1975). H295R cells were plated at the same density and stimulated the following day.

2.1.1 Cell passage and long term storage

For all cell lines, culture media was removed and cells washed with room temperature, calcium-free PBS. Trypsin-EDTA (0.5g/l trypsin, 0.2g/l ethylenediaminetetraacetic acid; Invitrogen) was added and the cells were returned to the incubator for 2 minutes. The reaction was quenched with standard growth media containing 10% FBS. The resulting suspension was centrifuged at 500g and the resulting pellet resuspended in growth medium for subsequent plating.

Confluent cells were trypsinised and centrifuged as described. The cell pellet was resuspended in a freezing solution of standard growth medium containing 10% DMSO (Dimethyl Sulfoxide; Sigma), and cooled at a maximum of 1ºC/min to -80ºC in 1ml cryotubules. These were transferred to liquid nitrogen for long term storage.

2.1.2 Cell stimulation

Cells were stimulated at 80-90% confluence. Media was removed and cells washed twice in PBS before stimulation. Cells were then incubated in either supernatant from a GIST

primary culture (GCM or NCM), or standard growth media supplemented with the following: Forskolin 10µM (Sigma); ACTH 1-24 100nM (Sigma); α-MSH (Sigma or Phoenix Pharmaceuticals); H89 10µM (Sigma). Media was removed after 24 hours and flash frozen prior to hormonal assay. Cells were washed twice with ice-cold PBS and removed for RNA extraction or melanin assay as described.

2.2 RNA extraction and RT-PCR

2.2.1 RNA extraction

Cells were washed twice with cold PBS, and lysed directly in the wells with the addition of 350µl buffer RLT from the RNeasy Mini Kit (QIAGEN). Well contents were scraped off and transferred to 1.5ml micro centrifuge tubes. RNA was extracted using the RNeasy kit according to the manufacturer's instructions. Fresh tissue specimens were collected in RNA Later (QIAGEN) and RNA extracted according to the RNeasy Mini Kit manufacturer's instructions as above.

2.2.2 Genomic DNA elimination

Genomic DNA was eliminated in a 50µl reaction containing 2µg RNA, 5µl 10x DNase Turbo buffer (Ambion), 0.5µl RNase inhibitor (40u/µl, Promega) and 1µl Deoxyribonuclease I (≥10,000U/mg, Ambion) and RNase free water (Qiagen) added to a final volume of 50µl, and incubated at 37ºC for 15 minutes. An equal volume of phenol pH 4.7 is added, the sample vortexed for 30 seconds then centrifuged at 14000 rpm for 1 minute. The upper aqueous layer is subjected to ethanol precipitation of RNA by the addition of 1/10 volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of 100% ethanol. After a 1-hour incubation at -80ºC samples were centrifuged at 14000rpm at 4ºC for 10 minutes, and the resulting pellet washed in ice-cold 70% ethanol. The centrifugation was repeated twice more, and the pellet allowed to air-dry.

2.2.3 First strand cDNA synthesis

Pellets were dissolved in 12.5µl RNase free water and 0.25µl random primers (500µg/l; Promega) added, before vortexing and incubation at 80ºC for 10 minutes, then cooled on ice. This was used for first strand cDNA synthesis at 37ºC for 1 hour in a reaction containing 4µl 5x MMLV RT-buffer (Promega), 2µl DTT (Dithiothreitol 0.1M), 1µl dNTPs

(25µM each of ATP, CTP, GTP, TTP; Promega), 0.5µl RNase Inhibitor (40u/µl), 1µl MMLV-RT enzyme (200u/µl; Promega) and RNase free water to final volume of 20µl.

2.2.4 Polymerase chain reaction

PCR was performed in a 20µl reaction containing: 1µl template DNA, 1µl 10µM F+R primers, 0.2µl 25mM dNTPs, 2µl 10X Standard Taq reaction buffer, 0.125µl Taq DNA Polymerase (all New England Biolabs). Thermocycling conditions were: initial denaturation 95ºC for 30s; denaturation 95ºC for 15s, annealling at 55-62ºC for 15s, extension 68ºC for 30s (30 cycles); final extension 68ºC for 5 minutes, holding at 4ºC. Human adrenal and skeletal muscle RNA was purchased from Clontech Laboratories (Cat No. 636528 and 636534) and cDNA synthesised as above. Human pituitary cDNA was obtained from Prof Marta Korbonits, human paraganglioma cDNA was obtained from Dr Sam O'Toole. THP-1 cDNA was kindly provided by Dr Rebecca Gorrigan.

2.2.5 Gel electrophoresis

Following PCR amplification samples were combined with 5µl of loading dye (Promega) containing 0.03% bromophenol blue and loaded onto a 1% agarose gel containing 0.05µl/ml GelRed (Biotium) within and electrophoresis chamber containing TAE buffer (Tris-acetate-ethylenediamene-tetraacetic acid buffer; National Diagnostics). An electrical current was passed through the tank and the cDNA visualised using a UVP UV transilluminator.

2.2.6 DNA gel extraction

The DNA fragment was extracted from the agarose gel using a clean scalpel, and DNA purified using the QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's instructions.

2.2.7 Primer sequences and annealing temperatures

GAPDH F TGC ACC ACC AAC TGC TTA G
R GGA TGC AGG GAT GAT GTT C, Ta 55-60ºC;
PC1/3 F TGT TCA CAC ATG GGG AGA GA
R ACG AGG CTG CTT CAT ATG CT, Ta 55ºC ;
PC2 F GAG AAG ACG CAG CCT ACA CC
R CCA TCA GCT TGC CCA GTA TT, Ta 55ºC;
POMC F AGT ACG TCA TGG GCC ACT TC

	R CTG ATT ATC TGC CAC GAC CC, Ta 60ºC;
MC1R	F ACT TCT CAC CAG CAG TCG TG
	R CAT TGG AGC AGA CGG AGT GT, Ta 64ºC
StAR	F CAA GGA GAT CAA GGT CCT GC
	R GAG GTC GAT TGC CTG AGT AGC C, Ta 64ºC
SCC	F GTC CTG TTG AAG AAG TCG GC
	R GAT GGA CTC AAA GGC AAA GC, Ta 64ºC

2.2.8 Real Time Quantitative PCR

Real time qPCR replicates a DNA template as in traditional, end point PCR, but quantifies this in real time by detection of a magnitude of fluorescence proportional to the amount of DNA amplified. A DNA binding dye SYBRGREEN is used which fluoresces when bound to dsDNA. This allows starting amounts of cDNA (and hence mRNA) to be calculated. For each reaction a Ct value is generated - the threshold cycle value at which amplification is first detected above background fluorescence. The lower the Ct, the greater the amount of amplification target in the starting cDNA. A standard curve of known DNA concentrations for each gene of interest (GOI) is used to calculate DNA copy number from Ct values. This is corrected for differences in the starting amounts of DNA with reference to a housekeeping gene, in this case GAPDH. A no template control without any starting DNA is used for quality control. Results were included if they met the following criteria: standard curve gradient = -3.3 (±0.3); efficiency 100% (±10%); R^2 = 0.97-1.00.

Quantitative PCR was carried out in a 10µl reaction containing 2µl cDNA template, 5µl 2x SYBRGREEN I (KapBiosystems), 0.2µl low Rox (KapaBiosystems), 0.5µl primers (10µM F+R) and 2.3µl RNase free water. The master mix contains the SYBRGREEN dye, $MgCl_2$, dNTPS, stabilisers and the DNA polymerase. Rox is a reference dye to control for evaporation during cycling.

Thermocycling was performed in a Stratagene MX4000 real-time thermocycler using conditions as follows: initial denaturation 95ºC for 3minutes; denaturation 95ºC for 3s, annealling at 55-62ºC for 30s, extension 68ºC for 1s (35 cycles); final extension 60ºC for 5 minutes.

2.3 Hormonal assays

2.3.1 Cortisol

Cortisol was assayed in serum and in cell culture supernatant using the Roche electrochemiluminescence immunoassay on the Elecsys 2010 analyser. Electrochemiluminescence is a kind of luminescence produced by electrochemical reactions in solutions. In this case the luminescence is produced by the application of an electrical current to a complex containing the element Ruthenium, which releases a photon at 620nm. The cortisol assay makes use of a competition test principle using a polyclonal antibody which is specifically directed against cortisol. Endogenous cortisol in the sample competes for binding with this antibody with a ruthenium complex labelled cortisol derivative. Depending on the concentration of the analyte in the sample and the formation of the respective immune complex, the labelled antibody binding site is occupied in part with sample analyse and in part with ruthenylated hapten. Streptavidin-coated microparticles are added, and the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into a measuring cell where the microparticles are magnetically captured on to the surface of an electrode. Application of a voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is generated by 2-point calibration within the analyser (Yaneva et al., n.d.) .

2.3.2 ACTH

ACTH was assayed in serum and culture medium using the Elecsys 2010 analyser, but this using the sandwich principle. Blood and culture supernatant are flash frozen on collection, and thawed for analysis. An aliquot of sample is added to a biotinylated monoclonal ACTH-specific antibody, and a monoclonal ACTH-specific antibody labelled with a ruthenium complex, and these react to form a sandwich complex. This becomes bound to the solid phase after the addition of streptavidin-coated microparticles, through the interaction of biotin and streptavidin. The reaction mixture is aspirated into a measuring cell and magnetically captured on to the electrode surface. A voltage is applied and the electrochemiluminescence emitted from the ruthenium complex measured as before.

2.3.3 Corticosterone

Corticosterone was assayed using the DEMEDITEC Corticosterone rat/mouse ELISA kit (Demeditecm DE4164). The microtitre wells are coated with a polyclonal antibody directed towards an antigenic site on the corticosterone molecule. Endogenous corticosterone in cell culture supernatant competes with a corticosterone-HRP conjugate for binding to the coated antibody. After incubation this is washed off. The amount of bound peroxidase conjugate is proportional to the concentration of corticosterone in the sample - after addition of substrate solution, the intensity of colour developed is inversely proportional to the concentration of corticosterone.

20µl of each sample or control (0-240nmol/l) are dispensed into appropriate wells, followed by 200µl of enzyme conjugate, and thoroughly mixed for 10 seconds. The mixture is incubated for 1 hour at room temperature, before the well contents are briskly shaken out. The wells are rinsed with 400µl of wash solution three times, with the plate being firmly struck on to absorbent paper each time. 100µl of substrate solution (tetramethylbenzidine, TMB) is added to each well and incubated for 15 minutes at room temperature, before the reaction is stopped by adding 50µl of "stop solution" (1 N acidic solution) to each well. The optical density was measured at 450nm using the Perkin Elmer Wallac Victor2 1420 microplate reader. A standard curve was generated using a 4 parameter logistic model with *myassays.com* and corticosterone concentrations in unknown samples determined. The dynamic range of the assay is between 1.63 - 240nmol/l and shows cross-reactivity with the following substances: progesterone 7.4%, deoxycorticosterone 3.4%, 11-dehydrocorticosterone 1.6%, cortisol 0.3%, pregnenolone 0.3%.

2.3.4 Alpha-MSH

Alpha MSH concentrations in cell culture supernatant was determined using two separate systems, the first an enzyme immunoassay (EIA) purchased from Phoenix Pharmaceuticals, the second a sandwich ELISA developed in collaboration with Dr Andrew Bicknell, University of Reading.

Alpha-MSH EIA: Microtitre wells are coated with a secondary antibody which can bind to the Fc fragment of a primary antibody, which in turn will be competitively bound by α-MSH in the samples, or a biotinylated α-MSH. The biotinylated peptide in the resulting

complex interacts with streptavidin-HRP, and this catalyses a reaction in the substrate solution (TMB) and a resulting colour change which is inversely proportional to the amount of α-MSH in the original sample. Peptide standard solutions are prepared with concentrations 0.01ng/ml - 1000ng/ml using a standard peptide supplied. Into each well is added 50µl of sample, standard or positive/negative control, 25µl of primary antibody and 25µl of biotinylated peptide, before a 2-hour incubation at room temperature (RT), with orbital shaking of 300-400rpm. Each well is then washed 4 times with 350µl of wash buffer, and emptied before adding 100µl of SA-HRP solution to each well for a 1-hour incubation at RT. Following this the wells are washed 4 further times as before. 100µl of TMB substrate is added and incubated in the dark for 1 hour at RT. The reaction is stopped with the addition of 100µl 2N HCL, changing the colour from blue to yellow. Optical density was read at 450nm and unknowns calculated as for corticosterone, outlined above.

Alpha-MSH Sandwich ELISA: Antibodies and ELISA plates were generated by Dr Andrew Bicknell, University of Reading as follows. Antibodies to alpha MSH were raised in sheep by cross-linking either the C-terminal (CRWGKPV-amide) or the N-terminal (Ac-SYSMEC-amide) of alpha MSH to purified protein derivative (PPD) using NHS-maleimide (Pierce). Animals were immunized on a monthly basis as described previously (Page, N. M. et al., 2003) and were titered using the same peptides linked to Maxisorb plates (NUNC). High affinity antibodies were subsequently purified using the same peptides linked to Ezlink Iodacetyl gel (Pierce) and the C-terminal antibody was labelled with biotin as described previously (Page, N. M. et al., 2003; Bicknell et al., 2001). 96 well Maxisorb plates (NUNC) were coated with 100ng/well of N-terminal antibody diluted in 0.1M sodium bicarbonate. The plates were subsequently blocked with blocking buffer (PBS containing 0.1% w/v bovine serum albumin (BSA)) and stored in the same buffer at 4ºC before use.

Plates were washed 3x with 300µl wash buffer (PBS containing 0.1% v/v Tween-20) before the addition of either standard or sample and left to incubate overnight at room temperature. The plates were subsequently washed 3X with wash buffer before the addition of 100ng/well of biotinylated C-terminal antibody diluted in blocking buffer. After incubation for 2hrs the plates were washed 3X with wash buffer before the addition of streptavidin-HRP conjugate (Sigma) diluted 1:2000 in blocking buffer. After a further incubation of 30 mins the plates were washed 3x with wash buffer before being developed

with TMB, the reaction stopped by the addition 1N HCL and the absorbance read at 450nm. Unknowns were interpreted from a four parameter logistic curve assay using myassays.com.

2.4 Melanin assay

The biological activity of synthetic α-MSH was tested on the basis of its ability to stimulate melanin production from B16F10 mouse melanoma cells. Cells were cultured as described in 6 well plates until confluent. Media was removed and cells were washed twice in PBS. Cells were stimulated overnight with 1μM ACTH 1-39 or 1μM α-MSH in standard media (DMEM plus 10% FBS), or supernatant from GIST primary culture. Culture media was removed and cells washed twice in PBS. Cells were detached with 200μl TE per well and the reaction quenched with 800μl of standard media. The cell suspension was pelleted by centrifugation at 500g, and resuspended in 1ml 1N NaOH. 200μl aliquots were added in duplicate to a 96 well microassay plate and absorbance measured at 420nm. Melanin concentration was determined with reference to a standard curve of synthetic melanin (Sigma) in the range 1-100μg/l, and corrected for the protein content of the cell lysate.

Protein content of the cell lysate was measured using a modified Bradford assay. Following a neutralization step using an equivalent volume of 1M HCl, 160μl aliquots of cell lysate were added in triplicate to a 96 well micro assay plate. 40μl of protein assay dye reagent (Bio Rad) was added to each well and incubated for 5 minutes at room temperature. Absorbance was measured at 595nm using a spectrophotometer. Protein concentration was determined with reference to a standard curve of bovine serum albumin (Sigma) in the range 0.1 - 1.0 mg/ml. Standard curves were plotted using Microsoft Excel.

2.5 General Histology

2.5.1 Paraffin embedding

Rat adrenals

After harvesting, rat adrenals were fixed immediately in neutral buffered formalin (Sigma) and incubated overnight at 4ºC. They were washed in H2O for 1 hour then dehydrated through a series of alcohols for one hour each at room temperature (50%,

70%, 90% and 100% ethanol) then cleared with 2 consecutive one hour incubations in xylene. Adrenals were then soaked in liquid paraffin for 24 hours at 56ºC with gentle agitation. The samples were poured into plastic moulds and left to set at room temperature.

Human tissue embedding

Human tissue specimens were processed using the Leica ASP300 S Fully Enclosed Tissue Processor prior to paraffin embedding using the Leica Histocore Arcadia Embedding system. FFPE processing of human paraffin sections was carried out by the pathology department of the Royal London Hospital.

2.5.2 Sectioning and Mounting

3µm sections were cut from paraffin embedded sections using the Thermo Shandon Finesse E+ microtome and mounted on TESPA treated slides (3-triethoxysilylpropylamine; VWR) using the floating out technique (Sack, 1963) on a water bath maintained at 45ºC. Sections were dried overnight at 37ºC to increase adherence.

2.5.3 Deparaffinisation

All staining techniques used required sections to be deparaffinised. Sections were deparaffinised in xylene for 2 changes of 2 minutes each, then the xylene was removed with 2 changes of 70% alcohol for 2 minutes each, before sections were placed under running tap water for 5 minutes.

2.5.4 Haematoxylin and Eosin Staining

Sections were deparaffinised as described, then stained in Gill's Haematoxylin (Sigma) for 15 minutes and washed in tap water for 5 minutes. Following a 1-minute incubation in 1% acid alcohol (70% alcohol; 1% conc.HCL) slides were washed in running tap water for 5 minutes, then stained with 1% Eosin (in distilled water) for 3 minutes. This was followed by a brief differentiation step in tap water, then rapid dehydration through an alcohol series (2 changes of 1 minute in 95% ethanol, 2 changes of 1 minute in 100% ethanol) then sections cleared with xylene (2 changes of 10 minutes). Sections were mounted with DPX mounting media (Lamb Laboratories) and covered with glass coverslips. Nuclei stain blue, other elements stain various shades of red. An optional 5 minute incubation in 5% potassium permanganate was used prior to initial haematoxylin

staining to bleach endogenous melanin - this process does not bleach lipofuscin (Hongwu Shen, 2015).

2.5.5 Periodic Acid Schiff Staining

Sections were deparaffinised as described, washed in tap water for 2 minutes then oxidised in 1% aqueous periodic acid solution for 5 minutes before a further 2-minute tap water wash. Staining was achieved with a 10-minute incubation in Schiff's reagent (Sigma), a ten-minute wash in tap water, a 2-minute incubation in Gill's haematoxylin for nuclear staining, a further 5-minute wash in running tap water. Staining was differentiated with a 1-minute incubation in acid alcohol, before a further 5-minute washing step in running tap water. Sections were dehydrated, cleared and mounted as described. Substances containing vicinal glycol groups of their amino or alkyl amino derivatives are oxidised by periodic acid to form aldehydes, which combine with Schiff's reagent to form an insoluble magenta compounds (Scott & Dorling, 1969). Nuclei stain blue.

2.5.6 Ziehl-Neelson Staining

Deparaffinised and rehydrated sections were placed on a staining rack and filter on carbol fuschin, and heated gently until steam started to rise. Sections were differentiated with 1% acid alcohol with alternate blotting until the sections were colourless, then washed in tap water for 10 minutes. Nuclei were counterstained in diluted methylene blue for 20 seconds, and sections dehydrated, cleared and mounted as before. Lipofuscin stains a strong purple pink by this method (Dayan et al., 1979).

2.5.7 Masson Fontana

Deparaffinised and rehydrated sections were impregnated in pre-heated silver solution at 60ºC checking every 5 minutes until the required intensity is obtained, before washing in distilled water for 1 minute, then incubating in gold chloride for 10 minutes, and washing in distilled water for a further minute. Staining was fixed by incubating in 5% sodium thiosulphate for 1 minute. Following a 10-minute wash in tap water sections were counterstained lightly in 1% neutral red, then dehydrated, cleared and mounted as before. For a working silver solution, concentrated ammonia is added drop by drop to 50ml of 5% Silver Nitrate until the precipitate just dissolves. More silver nitrate is added

until a faint opalescence persists. By this method melanin stains black, some forms of lipofuscin may also stain a brown/black colour (Bancroft & M, 2008).

2.5.8 Tissue Microarray

A tissue microarray was constructed using paraffin embedded adrenalectomy specimens from patients with primary aldosteronism - Conn's syndrome. For each patient, a H&E slide was used to identify the nodule and marked by a consultant histopathologist with a black marker. 0.6mm cores were extracted using the manual Beecher Instruments tissue arrayer. Three cores were taken from within the nodule, and three cores of "normal" adrenal cortex from outside the nodule were taken. Cores were added to open slots within a recipient TMA paraffin block that had been softened in an incubator for 10 mins at 37ºC. After this incubation the block was removed and the cores pressed inside with a glass slide to leave a smooth surface for sectioning. Sections were cut at 0.3µm as previously described and stained as for standard sections.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) was performed using three methods. Using the Envision Flex Peroxidase Kit (Dako) the secondary antibody is bound directly to the enzyme horse-radish peroxidase. In the presence of HRP, 3,3'diaminobenzidine (DAB) is converted to an insoluble brown pigment which can be visualised under bright field light microscopy. For certain antibodies, particularly those not raised in mouse or rabbit, it was more appropriate to use the Vectastain Elite Avidin-Biotin complex (ABC) method (Vector Labs) of antigen detection. Avidin is a tetrameric protein that binds with high affinity and specificity to biotin. In the ABC method the secondary antibody is conjugated to biotin to amplify the signal from the primary antibody. HRP is conjugated to a large avidin-biotin complex, and this is added to the sample to enzymatically label the primary - secondary antibody complex. This enzyme will change the colour of the DAB chromogen as before. Adding a conjugating streptavidin to alkaline phosphatase rather than HRP allows for the use of alternative chromogens.

All washing steps involved sections being covered in wash buffer (Envision Flex wash buffer; Dako) for 3 minutes, then rinsed briefly and covered similarly for two further changes. Antigen retrieval was then performed according to table X. Optimisation of

antigen retrieval conditions was performed for all antibodies according to the protocol described below. All antibody incubations were 30 minutes at room temperature. Counterstaining was performed by incubating for 30 seconds with Mayer's haematoxylin (Sigma) prior to a final dehydration, clearing and mounting with DPX.

2.6.1 IHC Methods

Envision Flex HRP IHC

Following antigen retrieval sections were washed as described, then incubated in Envision Flex peroxidase blocking reagent (Dako DM821) for 10 minutes at room temperature. After a second washing step, sections were incubated with primary antibody. Following a third washing step, bound primary antibody was visualised by incubating the slides for 30 minutes with Envision Flex/HRP (Dako DM822), washing and then developing using the 3,3'diaminobenzidine (DAB) chromogen. One drop of DAB chromogen per ml of ready-to-use stable substrate buffer (Biogenex) was mixed to prepare a working solution. This was applied to sections for 5 minutes, before washing in running tap water for 5 minutes. Sections were counterstained, dehydrated and mounted as described.

Linker

Staining using antibodies against CYP11B1 and CD117 also included a 10-minute incubation with Envision Flex+ LINKER following primary antibody incubation to amplify the signal.

Vectastain Elite ABC IHC

Sections were washed as above, then endogenous peroxidase activity was blocked with a 15-minute incubation in 3% hydrogen peroxide. A further wash step was followed by a 30-minute incubation in 2.5% horse serum, then application of primary antibody as above. Sections were washed, then incubated with a biotinylated secondary antibody (Table 2.1) for 30 minutes at room temperature. Following a further washing step sections were incubated in Vectastain ELITE ABC Reagent for 30 minutes at room temperature. After washing, the reaction product was developed using the DAB chromogen as described previously.

Streptavidin-AP

Staining performed as for ABC. Following biotinylated antibody incubation and washes, sections were incubated in streptavidin-AP (Vector Labs) for 30 minutes at room temperature. After washing the reaction product was developed using the Ultraview Universal Alkaline Phosphatase Red detection kit. Counterstaining with haematoxylin and mounting as before. This was used to provide a red staining for MITF on pigmented tissues when DAB might be non-specific (Jiang et al., 2014).

Avidin-biotin block

High levels of endogenous biotin in the adrenal gland may sometimes lead to significant levels of background staining using the ABC method. Where noted in table X, adrenal sections were consecutively incubated for 10 minutes with avidin and d-biotin solutions (Invitrogen 004303) prior to incubation with horse serum.

2.6.2 Antibody optimisation

For each new antibody used the specific conditions for antigen retrieval were optimised as follows. 12 sections of an appropriate control tissue were obtained from the histology library of the Core Pathology dept, Queen Mary, University of London. An appropriate staining technique (Flex HRP or Vectastain ABC) was selected.

Three different antigen retrieval solutions of varying pH were used:

- Vector antigen unmasking solution pH 6 (Vector Labs)
- Tris-EDTA Citrate Unmasking Solution pH 8.1 (EDTA 0.5g/l, Tris-Base 0.25g/l, sodium citrate 0.32g/l; adjusted to pH 8.1 with NaOH)
- Tris-EDTA unmasking solution pH 9.0 (EDTA 0.37g/l, Tris-Base 1.21g/l)

Sections were incubated in each of these antigen retrieval solutions in a waterbath at 95ºC for 10 or 40 minutes, or in a microwave set to full power for 10 minutes or 35 minutes. A starting primary antibody concentration was chosen based on the manufacturer's recommendations, or if these were not available, a starting concentration of 1/100 dilution was used. The antigen retrieval technique which provided the best balance between specificity and sensitivity of staining was chosen and the primary antibody concentration adjusted in turn. Additional steps were added as necessary (e.g. HRP linker incubation, or avidin/biotin blockade) to give a final protocol.

Table 2-1 Antibodies used in immunohistochemistry

Antibody	Manufacturer	Clonality	Antigen Retrieval	Primary Ab Concentration	Staining method
N-POC 1-28	AB Bicknell	Rabbit polyclonal	pH 9.0, 40 min water bath	1:2000	Flex
N-POC 1-49	AB Bicknell	Rabbit polyclonal	pH 9.0, 40 min water bath	1:2000	Flex
γ1-MSH	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:2000	ABC
γ3-MSH	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:1250	ABC
C-term ACTH	AB Bicknell	Rabbit polyclonal	pH 9.0, 40 min water bath	1:2000	Flex
Mid ACTH	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:1250	ABC
N-term ACTH	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:2000	ABC
N-term αMSH (acetylated)	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:1250	ABC
C-term αMSH (amidated)	AB Bicknell	Sheep polyclonal	pH 9.0, 40 min water bath	1:2000	ABC
HMB45	Dako	Mouse monoclonal	pH 9.0 , 40 min water bath	Ready to use	Flex
MC1R	Abcam ab125031	Rabbit monoclonal	pH 9.0 , 35 mins microwave	1:100	ABC
CYP11B1	Santa Cruz sc374096	Mouse monoclonal	pH 9.0, 40 min water bath	1:200 + linker	Flex
CD117	Dako 4502	Rabbit polyclonal	pH 9.0, 40 min water bath	1:1000 + linker	Flex
DOG-1	Leica NCL-L-DOG1	Mouse monoclonal	Sodium citrate buffer pH 6.0, 45 mins water bath	1:300	ABC
MITF	Ventana	Mouse monoclonal	pH 9.0 , 40 min water bath	Ready to use	Flex
S100	Abcam ab868	Rabbit polyclonal	pH 8.1 30 min microwave	1:200	ABC
Beta-catenin	Dako M3539	Mouse monoclonal	pH 9.0 , 40 min water bath	1:200	ABC
p27kip1	abcam ab3928	Rabbit monoclonal	pH 8.1, 40 min water bath	1:100	Flex
p57kip2	abcam ab4058	Rabbit polyclonal	pH 8.1, 40 min water bath	1:100	Flex
Ki67	Dako	Mouse monoclonal	pH 6.0, 40 min water bath	1:150	Flex
SDHB	Thermofisher 29843	Mouse polyclonal	pH 8.1, 40 min water bath	1:500	ABC
CYP11B2	C Gomez-Sanchez	Mouse polyclonal	pH 9.0 35 mins microwave	1:100	ABC

2.6.3 Immunocytochemistry

For immunocytochemistry, adherent cells were trypsinised and resuspended at 5 x 10^5 cells/ml. 200µl of cell suspension was added to a Shandon EX single cytofunnel and centrifuged at 800rpm for 5 minutes in a Shandon 3 cytospin centrifuge to deposit cells

on to a standard glass slide. Cells were fixed for 10mins in ice cold acetone, then staining proceeded as above, without any antigen retrieval step.

2.6.4 Slide Scanning and quantification of positive immunostaining

Slides were scanned using a NanoZoomer 2.0-HT Digital slide scanner and images analysed using NDP.View2 viewing software (Hamamatsu).

The Ki67 positivity index for zona glomerulosa and zona fasciculata was calculated as follows. ZG and ZF were identified histologically by H+E staining. Images from Ki67 stained sections were annotated to define these zones using the drawing facility of NDP.View2 viewing software. The total number of cells in each zone was counted manually. Cells exhibiting nuclear staining of Ki67, or those with mitotic figures were counted as positive by a single observer blinded to the treatments given. Mean percentage of Ki67 positive cells (+/- SEM) was recorded for each zone from each experimental condition in the rat adrenal remodelling experiment (see section 2.9). For other immunohistochemistry, interpretations of staining made by the author were confirmed by Professor Dan Berney, Consultant Histopathologist, Royal London Hospital.

2.6.5 Electron Microscopy

Paraffin embedded tissue was dewaxed in xylene and rehydrated through an alcohol series, incubated overnight in 0.2 M sodium phosphate buffer and fixed in 2.5% glutaraldehyde in 100mM phosphate buffer at pH 7.0 for three hours. Fresh surgical specimens were collected and fixed immediately in 2.5% glutaraldehyde. Specimens were postfixed in 1% osmium tetroxide in 100mM phosphate buffer for 2 hours at 4°C, dehydrated through a series of alcohols and propylene oxide using the Leica EM TP tissue processor and embedded in epoxy resin. 90nm sections were cut on the Reichert Ultracut E ultramicrotome. Samples were visualised using a J.E.O.L JEM1230 transmission electron microscope and images captured with an Olympus “Morada” digital camera.

2.7 Sanger sequencing

Genomic DNA (gDNA) extraction was performed from flash frozen tissue (DNeasy mini kit; QIAGEN) and whole blood lymphocytes (Illustra DNA Extraction kit; GE Healthcare) according to the manufacturer’s instructions. gDNA was extracted from paraffin blocks

using the QIAmp DNA FFPE Tissue Extraction kit (QIAGEN) according to the manufacturer's instructions. PCR was performed in a 20µl reaction containing: 1µl template gDNA (50ng/µl), 1µl 10µM F+R primers, 0.2µl 25mM dNTPs, 2µl 10X Standard Taq reaction buffer, 0.125µl Taq DNA Polymerase (all New England Biolabs). Thermocycling conditions were: initial denaturation 95ºC for 30s; denaturation 95ºC for 15s, annealling at 55-62ºC for 15s, extension 68ºC for 30s (30 cycles); final extension 68ºC for 5 minutes, holding at 4ºC.

Sequences corresponding to all exons from armadillo repeat containing 5 (ARMC5; NG_034258.1) were amplified by PCR following the protocol of Assié et al (Assie et al., 2013) and subjected to Sanger sequencing. The exon of the MC1R gene (NM_002386.3) and exons 1 and 2 of the SDHB gene (NM_003000.2) were amplified by PCR and similarly sequenced.

Sanger sequencing for the following genes was carried out within our own laboratory using primers as outlined in table X, then sequenced again by the genetics department at Royal Devon and Exeter NHS Foundation trust using gDNA extracted from tissues as described above.

1. Human adrenal and leucocyte DNA: Analysis of all coding regions and exon/intron boundaries of the PRKAR1A gene (NM_002734.3) and all coding regions and exon/intron boundaries of the PDE11A gene (NM_016953.3). Preliminary PDE11A sequencing was not carried out within our own laboratory.
2. GIST and leucocyte DNA: Sequence analysis of exons 9, 11, 13 and 17 of the KIT gene (NM_000222.2), exons 12, 14 and 18 of the PDGFRA gene (NM_006206.4) and codon 600 of the BRAF gene (reference sequence NM_004333.4). This assay can only detect a mutation present at a level of greater than 10% in a background of genomic DNA.

Sequences were analysed using CLC Sequence Viewer 7 (CLC Bio)

Table 2-2 Details of primers used for Sanger Sequencing

	Forward Primer	Reverse Primer	Ta °C
c-Kit exon 9	TCCTAGAGTAAGCCAGGGCTT	TGGTAGACAGAGCCTAAACATCC	58
c-Kit exon 11	GTGCTCTAATGACTGAGAC	GTGCTCTAATGACTGAGAC	58
c-Kit exon 13	GACATCAGTTTGCCAGTTGT	TGTTTTGATAACCTGACAGAC	58
c-Kit exon 17	TGTGAACATCATTCAAGGCG	GACTGTCAAGCAGAGAATGGG	58
PDGFRA exon 12	TCCAGTCACTGTGCTGCTTC	GCAAGGGAAAAGGGAGTCTT	60
PDGFRA exon 14	TCTGAGAACAGGAAGTTGGTAGC	CCAGTCAAAATCCTCACTCCA	60
PDGFRA exon 18	ACCATGGATCAGCCAGTCTT	TGAAGGAGGATGAGCCTGACC	60
BRAF exon 15	ATCTCTTACCTAAACTCTTCATAATGC	AACTCAGCAGCATCTCAGGG	58
hSDHB exon 1	TTGGATATTGAATGCCTGCC	TTGGTCTTGAACTCCCAACC	60
hSDHB exon 2	TCCGAAGGTGACCTGAGAAG	CTCTATCAGCTTTGGCCAGC	60
PRKAR1A exon 1	CCACCTGTCATCTGAGACGC	CCGGTTCTCTCCTCCTTCC	62
PRKAR1A exon 2	AATGCCAGATTGACATTTTGC	AATCACCTCATCATCTCCCC	62
PRKAR1A exon 3	GAGTGCCAGCTTTACATGCC	ATGACCACCAAGTGGGTCC	62
PRKAR1A exon 4	TAATTGAAGCGCAGGTTGC	AACAATTTGGGATCACACCC	62
PRKAR1A exon 5	GCTTAATGTTTGAAATTCACGG	ATATTGCATGCTCCAGAGGC	62
PRKAR1A exon 6	TTGCTTGATTTTCTTTCCCC	ATTCTTATTGCTCGGAAGCG	62
PRKAR1A exon 7	TTTTTGATGTCACTTGCACTTT	TTCTAAATCACACTCTCAAACACCA	62
PRKAR1A exon 8	TGATAATTACACGTCTTGGGG	GCTTTTCCCAAGTCCATCC	62
PRKAR1A exon 9	AGAATGTTGAATGGGCATGG	GAATTAGCCCACTCTTTCCC	62
PRKAR1A exon 10	GTTTAAGGTGCCACCCTGG	GAAACCATGTATTTATTGTGAGGG	62
PRKAR1A exon 11	AGCCTGTTACCCATCTTTGC	GCAGTTTGCATGAGTGAAGC	62
MC1R	ATCCTTCCTGGACAGGAC	CAT TTA GTC CAT CCT CTT TG	62
ARMC5 exon 1 (a)	GATTCTCCCTCGCCTCTTCT	GAGCTTCTCACGCCTACCTC	60
ARMC5 exon 1 (b)	GTCGGACTTCTGGGCTGTTC	CTCAGGGGTGTCTCGTTGGT	60
ARMC5 exon 1 (c)	TTTCCCTGTCTTCCAGTTCC	ACGTTATTCCGGGATAGGAC	56
ARMC5 exon 2	AGGGGTAGAACCCTCACAAG	CACTCAAGCCTTTCTTCTGC	60
ARMC5 exon 3 (a)	GTAAGAGGCTGTGAGGTTGG	TCATCTACCAGCACCTCCAC	64
ARMC5 exon 3 (b)	GATCCTCGCCAACCTGTGT	CACTGCTCACCCTCCCAAG	60
ARMC5 exon 4 (a)	TTGGCTCTGGGTTCAGTCTC	GAGTGGGAAGGTGAGGTTCT	60
ARMC5 exon 4 (b)	GGCCTGCTGACCTATGTGAC	CAGAAGGGCTCCTTGGTCTA	60
ARMC5 exon 5	GTCTCACTCACCCCACCTG	ACAGTGGGAGACACCAGGTC	60
ARMC5 exon 6 (a)	ACACCCGCTCTTCCTCTTCT	CTCTTCCAGCTCCTCCTCCA	60
ARMC5 exon 6 (b)	CTGCTGGCCGTTTCCTACTG	GAACAAGACCCTGCTTGGTG	60

2.8 Pyrosequencing

The role of methylation status of the POMC promoter in enabling POMC expression has been established by Newell Price *et al* (Newell-Price et al., 2001). They demonstrated that the POMC promoter is embedded within a defined CpG island, much of which is methylated in unexpressing tissues, and specifically unmethylated in POMC-expressing tissues and tumours. Ye *et al* (Ye et al., 2005) defined a region between -417 and -260 bp upstream of the POMC transcription initiation site where the levels of POMC expression correlated inversely with methylation density. Methylation levels at this site were assessed using bisulphite modification of genomic DNA and pyrosequencing.

2.8.1 Principle of methylation analysis

Methylation of any of the four nucleotides making up DNA can occur in various nucleotide pairs, with the most common being methylation of the cytosine residue in the context of a CpG dinucleotide. Conversion of this epigenetic change into a genetic change allows it to be measured by DNA sequencing. Incubation of the DNA sample with sodium bisulphite converts unmethylated C into U (Uracil) while leaving methylated C unmodified. PCR primers may be designed that are specific to the modified DNA strand. Any C which is not followed by a G is now treated as a T, since uracil will be converted to thymine during PCR; one of the primers is tagged at the 5' end with a biotin label. Primers should ideally be designed which generate a PCR product that is 150-250 base pairs, without primer overlap onto CpG sites.

Streptavidin beads are combined with the PCR product to select out the biotin labelled product, then the DNA is denatured so that single stranded molecules may be sequenced. Pyrosequencing uses a chemical light reaction to detect the incorporation of dNTPs into the synthesising strand. A sequencing primer is hybridised to the amplified DNA template and incubated with DNA polymerase, ATP sulfurylase, luciferase and appears, along with the substrates, adenosine 5' phosphosulphate (APS) and luciferin. The first of four dNTPs is added to the reaction, and if complementary it is incorporated into the growing strand, accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. PPi is converted to ATP by ATP sulfurylase in the presence of APS, and this ATP drives luciferase-mediated conversion of luciferin to oxyluciferin, generating levels of visible light proportional to the amount to ATP. The light is detected

and recorded as a peak in a program which is proportional to the number of nucleotides incorporated.

Apyrase degrades unincorporated nucleotides and ATP, and the next nucleotide is added when degradation is complete. dNTPs are added one at a time and the process continues, with the sequence being determined by the light generated with each dNTP addition. The degree of methylation at CpG sites is determined according to the ratio of T and C at that point.

2.8.2 Pyrosequencing method

Primers were designed using the PyroMark Assay Design Software 2.0 to include a region -417 to -260 upstream of the POMC transcription initiation site.

Amplification primers.
POMC_Meth_Forward AGGAGGGAGTAGAAGTTAGG
POMC_Meth_Reverse [Btn]AACATCACCCTCTCCCCATTTA

Sequencing primers
POMC_Sequencing_Primer_1: GGGAGTAGAAGTTAGGA
POMC_Sequencing_Primer_2: GTTTAGGGGAGGTTTTT

Genomic DNA was extracted from GIST tumours and lung carcinoid tumours associated with and without Cushing's syndrome, and pituitary corticotroph adenoma as described previously. gDNA was initially quality controlled using Nanodrop 8000 UV-Vis Spectrometer and Tapestation 2200. 500ng of each Genomic DNA sample were bisulphite converted using the EZ-96 DNA Methylation Kit (Zymo Research) as described by the manufacturer's protocol alongside a control DNA sample (CEPH). Samples were then quality controlled using the Nanodrop 8000 UV-Vis Spectrometer to check concentrations of resulting bisulphite converted DNA. Converted DNA was diluted to 5ng/μl ready for PCR reaction. Primers POMC_Meth_Forward and POMC_Meth_Reverse were then used initially to test the Control sample (annealing temperature gradient) and then subsequently on the samples themselves. The reverse primer was biotinylated for this PCR reaction and subsequent PCR products were run on a 1.5% agarose gel to access the product, including a negative.

The MyTaq PCR (Bioline) kit was used in a 50μl reaction: 10μl Reaction Buffer, 1μl POMC_Meth_Forward, 1μl POMC_Meth_Reverse, 0.5μl Taq Polymerase, 35.5μl Water, 2μl of diluted bisulphite converted DNA (5ng/μl). Thermocycling conditions for PCR were: 95°C for 1 minute; 95 for 15 seconds, 50°C for 15 seconds, 72 for 10 seconds (25 cycles).

20μl of the PCR products were added to 37μl of Binding Buffer and 3μl of Streptavadin beads, then shaken for 20 minutes at 1600rpm. Using a vacuum preparation workstation samples were washed with 70% ethanol, 0.2M NaOH and wash buffer. Samples were then transferred into 43.5μl of annealing buffer and 1.5μl of sequencing primer (POMC_Sequencing_Primer1 or POMC_Sequencing Primer2). Samples were heated for 2 minutes at 80°C and then placed onto the PyroMark Q96 ID for analysis. Control DNA was obtained from Thermofisher Scientific (CEPH Individual 1347-02).

2.9 Rat adrenal remodelling

Adult male Wistar rats of 8 weeks' old weighing 100 - 120g were obtained from Charles River Laboratories. They were housed in rooms with controlled light and temperature and treated under the Home Office Animals (Scientific Procedures) Act 1986 - project licence 70/6841 "Mechanisms underlying adrenocortical growth". Animals were kept under conditions expected to stimulate or inhibit remodelling of the zona glomerulosa as described by Brennan *et al* (Brennan et al., 2008). Control animals were maintained on a standard diet consisting of dry pellets made from wholemeal flour (Sainsburys Ltd, London, UK) supplemented with 1% CaCO3 (Sigma) and 1% NaCl (Sigma) with access to distilled water for two weeks. In addition, one group of animals received the standard diet with supplementation of their drinking water with Captopril (0.5mg/ml; Sigma) and one group received a low sodium diet which omitted the 1% NaCl.

Animals were anaesthetised in a CO_2 chamber, then killed by cervical dislocation. 3 animals from each group were sacrificed at days 2, 4 and 7. At this point the groups were crossed over: captopril treated animals were converted to a low sodium diet; low sodium diet animals were given a standard diet with captopril supplementation. 3 animals per crossover group were sacrificed at days 9, 11 and 14. The left adrenal from each animal

was fixed overnight in neutral buffered formalin (Sigma) and processed as described previously. The right adrenal was embedded in Tissue-Tek OCT Compound (Sakura) and flash frozen on dry ice, then stored at -80ºC.

2.10 Dynamic clinical investigations

Low dose dexamethasone suppression test.

A baseline serum cortisol level is taken at 9am, then 0.5mg oral dexamethasone is administered immediately and every 6 hours for 8 doses (i.e. 0900h, 1500h, 2100h and 0300h). Bloods are taken for cortisol assay at 24 and 48 hours. Serum cortisol should be suppressed to <50nmol/l. Failure to suppress is seen in the autonomous production of cortisol in Cushing's syndrome, but also in certain other conditions associated with altered cortisol dynamics (e.g. depression, obesity). The test should therefore be interpreted in conjunction with the clinical suspicion for Cushing's syndrome.

Mixed Meal Test

Following a baseline 9am assessment of fasting serum cortisol, a meal comprising 30g cornflakes, 250ml semi-skimmed milk, 4.5g sachet sugar, 170ml unsweetened orange juice, 40g (medium) slice white bread, 10g portion butter, 50g piece cheddar cheese and 100g low fat fruit yoghurt (composition 31.1g Protein / 124.4 kcal / 16%, 31.1g Fat / 297.9 kcal / 38% and 95.6g CHO / 358.5kcal / 46%). Blood samples were then obtained post prandially at +15, 30, 45, 60, 90 and 120 minutes. The test is performed to assist in the diagnosis of food-dependent Cushing's syndrome, a rare condition due to the ectopic expression of receptors for gastric-inhibitory polypeptide on the surface of adrenocortical cells (Christopoulos et al., 2005). A rise in cortisol levels following the meal is suggestive of the syndrome.

Octreotide Test.

Following a baseline 9am assessment of fasting serum cortisol, a 100mcg subcutaneous dose of octreotide is administered, and repeat measurements of serum cortisol taken hourly for the next 8 hours. Octreotide is a somatostatin analogue, and is used to inhibit the function of various neuroendocrine cell types expressing somatostatin receptors. The intention with this octreotide test dose is to transiently reduce the production of a potential stimulating factor from an occult or apparent neuroendocrine tumour. This

would result in a transient fall in serum cortisol levels in the hours afterwards (Libe et al., 2010).

CRH Test

Three basal fasting samples of cortisol are taken at 15 minute intervals. Immediately following the third sample 100mcg of corticotropin releasing hormone is administered intravenously. Further serum samples are taken for cortisol assay at intervals up to 120 minutes' post-injection. A rise in cortisol from basal to peak of >20% suggests a pituitary source. A rise in ACTH from basal to peak of > 50% suggests a pituitary source (Kaye & Crapo, 1990).

2.11 Data Analysis

All data were analysed using Microsoft Office Excel for Mac 2011 and SPSS version 22. One-way ANOVAs (analysis of variance) were implemented, and the appropriate pre-planned and post-hoc comparisons including the bonferroni correction for multiple comparisons(Brace et al., 2006). Pre-planned tests are those between factors that are expected to differ, and are already decided upon before carrying out an experiment. Post-hoc comparison tests are comparisons not foreseen, that may reveal interesting connections between data sets. Graphs were produced using SPSS version 22 and Autodesk Graphic for Mac.

2.12 Ethics and consent

Ethical approval for studies involving human tissue was granted as follows.

Consent was taken for experiments involving GISTs, human adrenal primary culture, human adrenal from ACTH-dependent Cushing's disease, PPNAD adrenal specimens, lung carcinoid tumours:

Genetics of Endocrine Tumours
Reference: 06/Q0104/133
Huntingdon Research and Ethics Committee, 14 Feb 2007

Experiments involving human adrenal tissue taken at the time of adrenalectomy for Conn's syndrome:

Analysis of bone morphogenetic protein and sonic hedgehog signal transduction pathway components in adrenal tumours and adjacent normal tissue
Reference: O8/H0701/59
Redbridge and Waltham Forest Local Research Ethics Committee, 8th August 2008.

Chapter 3 The role of p27kip1 and p57kip2 in rat adrenal remodelling

3.1 Aims

This chapter aims to understand mechanisms controlling remodelling of the rat adrenal gland. Using dietary and pharmaceutical modification of the renin-angiotensin-aldosterone system the adrenal response will be assessed in terms of histological changes, and immunohistochemical expression of markers of proliferation and the cell cycle.

Rats will be placed on a low sodium diet to activate the renin-angiotensin-aldosterone system (RAS), or treated with an ace-inhibitor in their water supply to inhibit the RAS. The adrenal response to these experimental conditions will be assessed in terms of the gross histological changes within the ZG, and by expression of the Ki67 marker of cell proliferation by immunohistochemistry. The expression of the cyclin-dependent kinase inhibitors p27kip1 and p57kip2 will also be assessed by immunohistochemistry. There is a literature to suggest that these may mark populations of progenitor cells (Bilodeau et al., 2009; Besson et al., 2007), and changes in their expression during rapid ZG remodelling may indicate the locations of these cells.

3.2 Introduction

3.2.1 Adrenal remodelling

The response to changes in activity of the HPA axis or RAA system in terms of steroid production are rapid. In rodent models RAAS activation in low sodium conditions rapidly expands the ZG, with an increase in the number of CYP11B2 expressing cells and therefore more aldosterone secretion (Romero, Yanes et al., 2007). Similar changes have been demonstrated in patients with primary aldosteronism (Boulkroun et al., 2010). On the other hand, suppression of the RAAS by administration of angiotensin-converting enzyme (ACE) inhibitors causes rapid shrinking of the ZG, a reduction in CYP11B2 expression(Romero, Rilli et al., 2007). Likewise, excessive ACTH stimulation leads to expansion of the ZF and increased CYP11B1 expression, and the converse in response to suppression of pituitary ACTH release (Dallman, 1984).

There are several possible mechanisms by which such remodelling could occur. Potentially this could involve increasing rates of differentiation of stem or progenitor cell populations, or it could arise from proliferation within existing cell populations - enhanced proliferation has been shown within the ZU and the ZF or ZG depending on which is being remodelled(McEwan et al., 1996). There may be transdifferentiation from one steroidogenic population to another, for example ZF cells converting to ZG cells when the RAAS is activated, or dedifferentiation of ZG cells when the RAAS is inhibited. Changes in the rate of apoptotic cell death may also be altered – there is evidence that ACTH forestalls apoptosis of terminally differentiated ZR cells in the hypophysectomised rat (Carsia et al., 1996) How these processes are controlled is unclear.

3.2.2 Control of cell cycle progression

Regulation of cell cycle progression is an important determinant of cell proliferation and differentiation. Progression though the cell cycle is controlled by cyclins and cyclin-dependent kinases (CDKs). CDK activity is regulated by a variety of mechanisms, including intracellular levels of cyclin, the presence of CDK inhibitors, or phosphorylation of the CDKs themselves.

The cyclin-dependent kinase (CDK) inhibitors can be divided into two main families: INK4 and Cip/Kip. The INK4 gene family encodes p16INK4a, p15INK4b, p18INK4c and p19INK4d. All of these bind to CDK 4 and CDK6 and interfere with their binding to D-type cyclins, thereby inhibiting their kinase activities (Sherr & Roberts, 1995). The Cip/Kip family members p21Cip1, p27kip1 and p57kip2 have an identical N-terminal domain which binds and inhibits a variety of cyclin-CDK complexes, with their primary target being the cyclin E-cdk2 complex (Gomez Lahoz et al., 1999; Musgrove, 2006) This complex is of particular importance in regulating the G1/S transition of the cell cycle.

The roles of p57 and p27 in the development of the adrenal gland have been shown in knockout models. In the mouse adrenal gland, p27 expression was demonstrated in the medulla but not the cortex, and p57 in the cortex but not the medulla (Nagahama et al., 2001). Mice lacking p27 had a phenotype suggesting impaired control of cellular proliferation, with an increase in size of the adrenal medulla, as well as the thymus, testis and pituitary, although without gross developmental defects (Nakayama et al., 1996) Knockout of p57 leads to death shortly after birth from the respiratory consequences of a

cleft palate, but these animals are noted to have adrenocortical hyperplasia (Zhang et al., 1997).

The effect of ACTH on the expression of p27 and p57 in the rat adrenal was investigated by Kobayashi *et al* (Kobayashi et al., 2006). They showed low expression of p27 in the rat cortex, in keeping with the earlier descriptions in the mouse. This was present mainly in the ZG, and was diminished by suppression of ACTH stimulation of the gland using high dose dexamethasone. Expression of p57 expression was not altered by dexamethasone, but was increased by treatment with ACTH, and again this was recognised mainly within the zona glomerulosa.

3.2.3 Quantification of proliferation using Ki67

Ki67 is a nucleolar non-histone protein identified in the 1980s, which is universally expressed in proliferating cells but absent in quiescent cells. It is therefore used widely as a marker of tumour proliferation, where it has been shown to have both prognostic and predictive value. It functions as a cell-cycle regulated protein phosphatase 1 binding protein, and is involved in the assembly of the perichromosomal compartment during mitosis (Booth et al., 2014) It also has a role in spatially organizing heterochromatin to enable specific expression of genes required for proliferation (Sobecki et al., 2016).

Its exact function is not known, but it is thought to be involved in the synthesis of ribosomal RNA (Scholzen & Gerdes, 2000). The fraction of Ki67 positive cells in a tumour sample correlates well for many cancers with the course of the disease, and it is now well established as a factor in scoring systems to determine the prognosis of adrenocortical cancer (Terzolo et al., 2001)

It has also been used widely in experiments investigating adrenal remodelling to identify dividing cells. In response to chronic stress increased Ki67 density has been demonstrated in the outer zona fasciculata of the rat adrenal (Ulrich-Lai et al., 2006), during compensatory growth following unilateral adrenalectomy (Engeland et al., 2005), or regrowth following adrenal enucleation (Ennen et al., 2005).

3.2.4 Hypothesis

It was hypothesised that as controllers of cell cycle progression p27 and p57 would mark transiently amplifying populations of progenitor cells during differentiation of stem cells, and thus might indicate the location of these populations. By forcing the gland to undergo rapid differentiation during remodelling these changes would be more obvious.

3.3 Results

3.3.1 Experiment design

8-week old adult male Wistar rats were maintained under conditions expected to stimulate or inhibit the RAS. Control rats received a diet of wholemeal flour supplemented with 1% CaCO3 or 1% NaCl. RAS activation was achieved by omitting the NaCl from their diet, while captopril was added to drinking water to inhibit the RAS.

Three rats from each experimental group were sacrificed at 7 days and 14 days. At 7 days 3 rats on low sodium diet switched over to RAS inhibition with captopril and a normal diet, and 3 rats receiving captopril had this stopped, and were placed on to a low sodium diet (Figure 3.1). This was performed to equalise the basal level of RAS activation (maximally activated or inhibited) across each group. Additionally, the stimulus each experimental group achieved would be of a greater magnitude, thereby amplifying any effects in terms of changes in expression of cell cycle markers. The left adrenal of each animal was harvested and stained as described for H&E, Ki67, p27kip1 and p57kip2. (Full details can be found in Section 2.9)

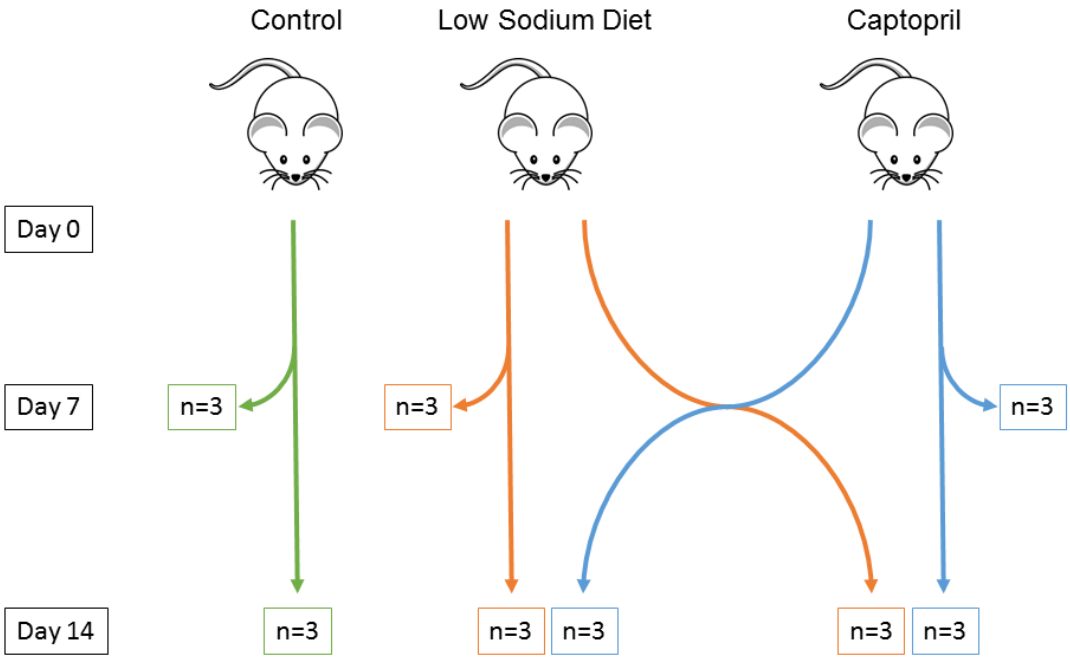

Figure 3.1. Experimental design. Rats were fed a normal diet, a low sodium diet, or a normal diet supplemented with captopril in their drinking water. Rats were sacrificed at 7 days and 14 days. At 7 days 3 rats from each experimental groups were crossed over to receive the reverse treatment.

3.3.2 Activation of the renin-angiotensin aldosterone axis causes expansion of the zona glomerulosa

The histological identity of the ZG was determined using standard H&E staining. Rats fed a low sodium diet demonstrated expansion of the ZG from a thickness of 8-10 cells below the capsule to 14-17 cells (Fig 3.5 panel C). Captopril treatment led to a slight contraction of the ZG to 6-8 cells below the capsule (Fig 3.5 Panel G). Crossing over from low sodium to captopril resulted in ZG contraction (Fig 3.5 Panel H), and crossing over from captopril to low sodium caused ZG expansion as above (Fig 3.5 Panel D).

An attempt at functional identification of the ZG using immunohistochemical labelling for CYP11B2 was initially successful (Figure 3.2), demonstrating that RAS activation increased CYP11B2 expression and captopril inhibited it. However, this same antibody failed to successfully stain positive controls reliably, and we were unable to use it for all sections. Alternative markers for this zone include the angiotensin II receptor, but no antibody was available. The use of β-catenin to label ZG (Drelon et al., 2015) was attempted but unfortunately staining could not be well optimised. Although there is

increased β-catenin staining in the same region as CYP11B2 in initial controls, the staining in general is difficult to interpret, and therefore was not used in the formal analysis.

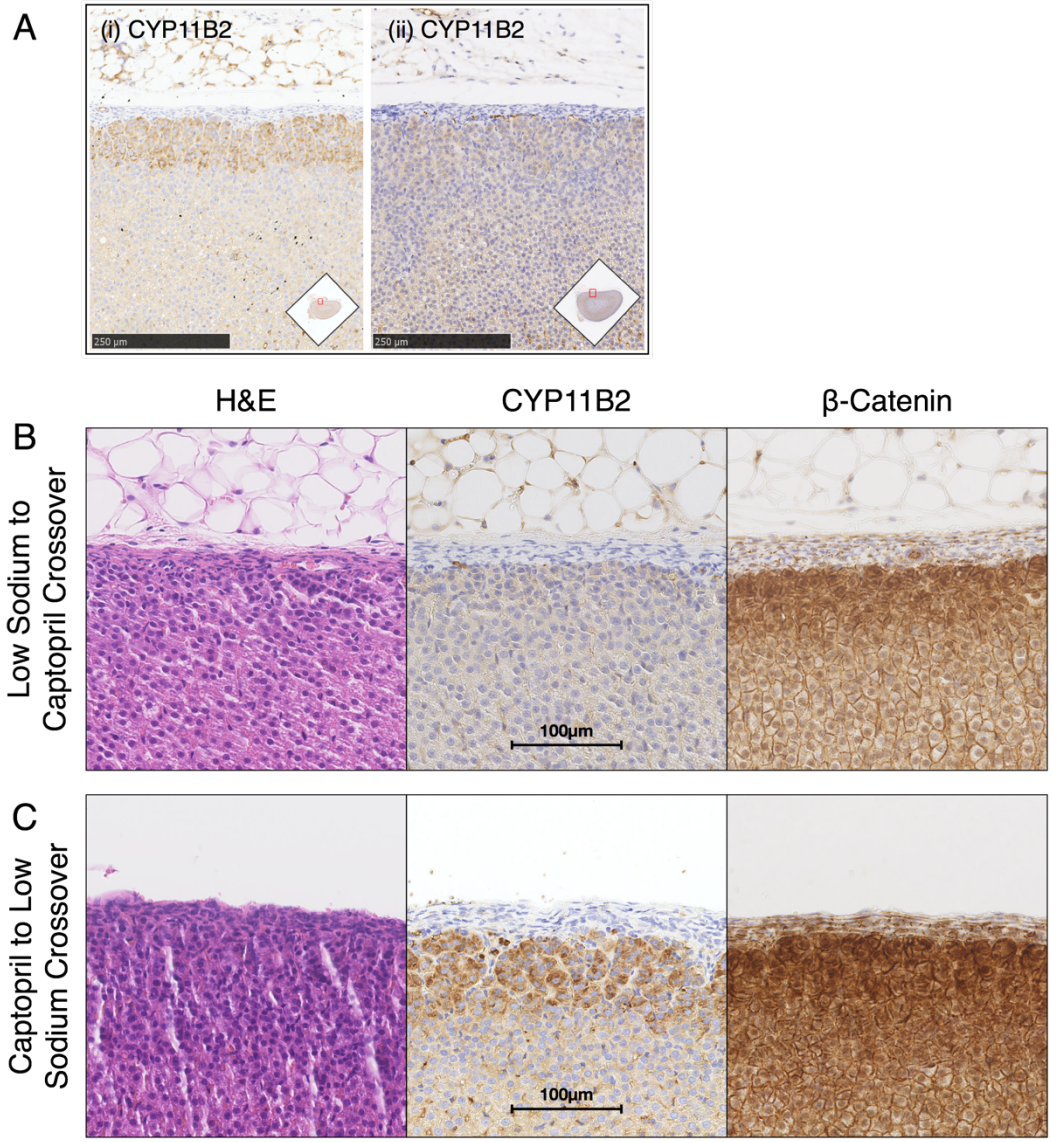

Figure 3.2 CYP11B2 and β-catenin staining in the rat adrenal gland following 7 days of low sodium diet or captopril treatment, followed by 7 days of the opposite treatment. Sections were stained for CYP11B2 (1:100) and beta-catenin (1:200). Maximal inhibition of the renin-aldosterone system (RAS) using captopril, followed by 7 days of RAS stimulation on a low sodium diet results in CYP11B2 expression in the zona glomerulosa. There is increased beta-catenin expression but overstaining with this antibody in basal conditions makes this impossible to reliably interpret. Images were taken using the NanoZoomer 2.0-HT Digital slide scanner. Scale = 100μm. See Section 2.6.3 for full method. Panel A shows the same positive control adrenal stained for CYP11B2 on separate occasions, showing ZG staining in the left panel (i), and lack of staining in the right panel (ii).

3.3.3 RAS activation causes an increased in proliferation within the ZG

Sections were immunostained for Ki67, a marker of proliferation. ZF and ZG were identified using H+E sections, and identified on Ki67–stained slides using drawing software on the NDP.View2 viewing programme. All cells were counted within each zone across the entirety of the section, and mean percentage (+/- SEM) of Ki67 positive cells for each condition is shown in Figure 3.3 (See Section 2.6.4). Proliferation as assessed by Ki67 positivity was low in all zones of the adrenal cortex in control animals. Rats fed a low sodium diet to stimulate the RAS demonstrated increased proliferation in the ZG at 7 days, and greater still at 14 days (Figure 3.5 Panel C). At 7 days most of the Ki67 positive cells were just below the capsule (Figure 3.5 Panel B), but at 14 days and in the animals crossing over to low sodium diet, Ki67 positive cells were spread throughout the ZG (Figure 3.5 Panel D). Captopril treatment significantly reduced the number of Ki67 positive cells in the ZG (Figure 3.3), but in animals treated with captopril for 14 days, or treated initially with low sodium diet and then switched to captopril, there were Ki67 positive cells at the border of ZG/ZF (Figure 3.5 Panels G and H). There was no significant change in the Ki67 proliferative index within the ZF in any of the experimental groups (Figure 3.2).

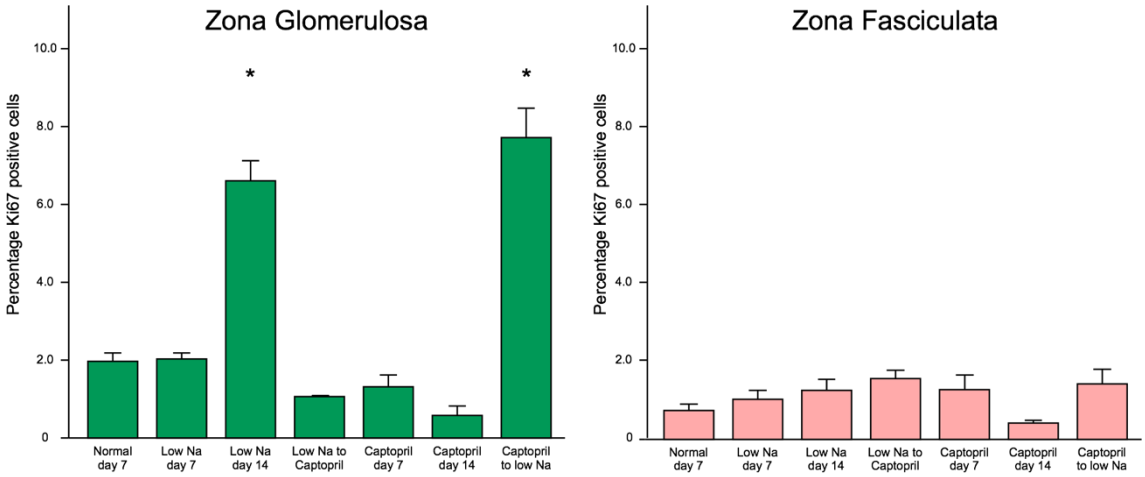

Figure 3.3. Ki67 proliferative index in ZG and ZF cells under RAS remodelling conditions
Rats were fed a normal diet, a low sodium diet, or a normal diet supplemented with captopril in their drinking water. Rats were sacrificed at 7 days and 14 days. At 7 days 3 rats from each experimental groups were crossed over to receive the reverse treatment. Sections were immunostained for Ki67, a marker of proliferation. ZF and ZG were identified using H+E sections, and identified on Ki67 –stained slides using drawing software on NDP.View2 viewing programme. All cells were counted within each zone across the entirety of the section, and mean percentage (+/- SEM) of Ki67 positive cells) for each condition is shown. A significant increase in expression was found in the adrenal from rats who were treated with a low sodium diet to activate the RAS for 14 days, or in rats that were initially treated with captopril to inhibit the RAS. * = $p<0.05$ (using one-way ANOVA as described in section 2.11)

3.3.4 Staining pattern of p27 and p57 in control animals.

Nuclear p27 staining was detected in the medulla, and the subcapsular cortex in control animals (Figure 3.4 Panel A). In addition, there were p27 positive cells in the adrenal capsule. Nuclear staining for p57 was detected throughout the cortex, with the greatest degree of staining in the ZG (Figure 3.4 Panel C). There was no p57 staining in the medulla.

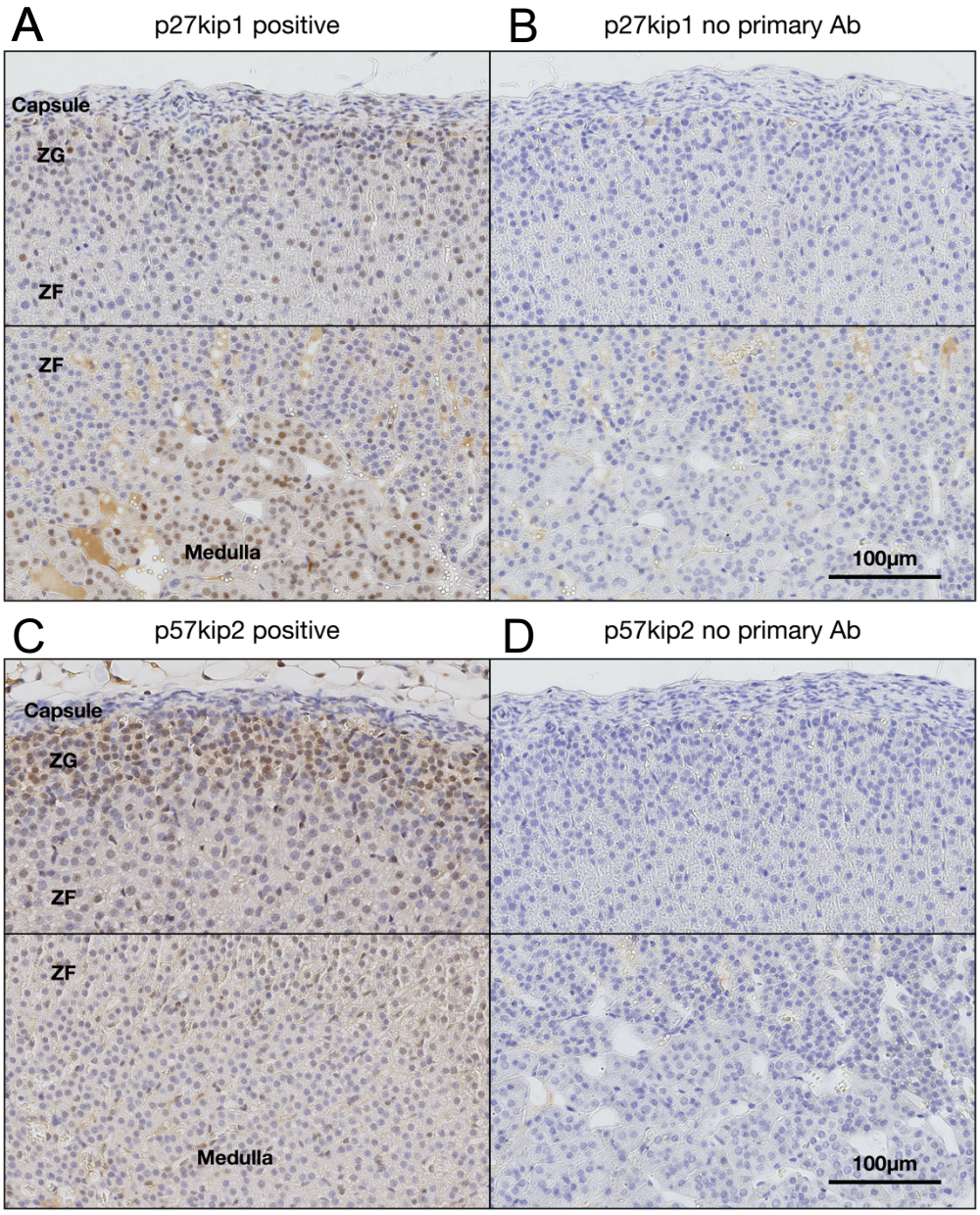

Figure 3.4 p27kip1 and p57kip2 staining in control adrenals
Control sections of rat adrenal gland were provided by the tissue library of the Core Pathology Dept, Blizard Institute, QMUL. Immunohistochemical staining was carried out for p27kip1 and p57kip2 (Ab concentrations 1:100; see section 2.6.2). No antibody controls shown in B and D. Positive nuclear immunostaining for p27 is seen in the adrenal medulla and the outer cortex (A). Staining for p57 is not present in the medulla, but is found throughout the cortex and is particularly strong in the ZG (C)

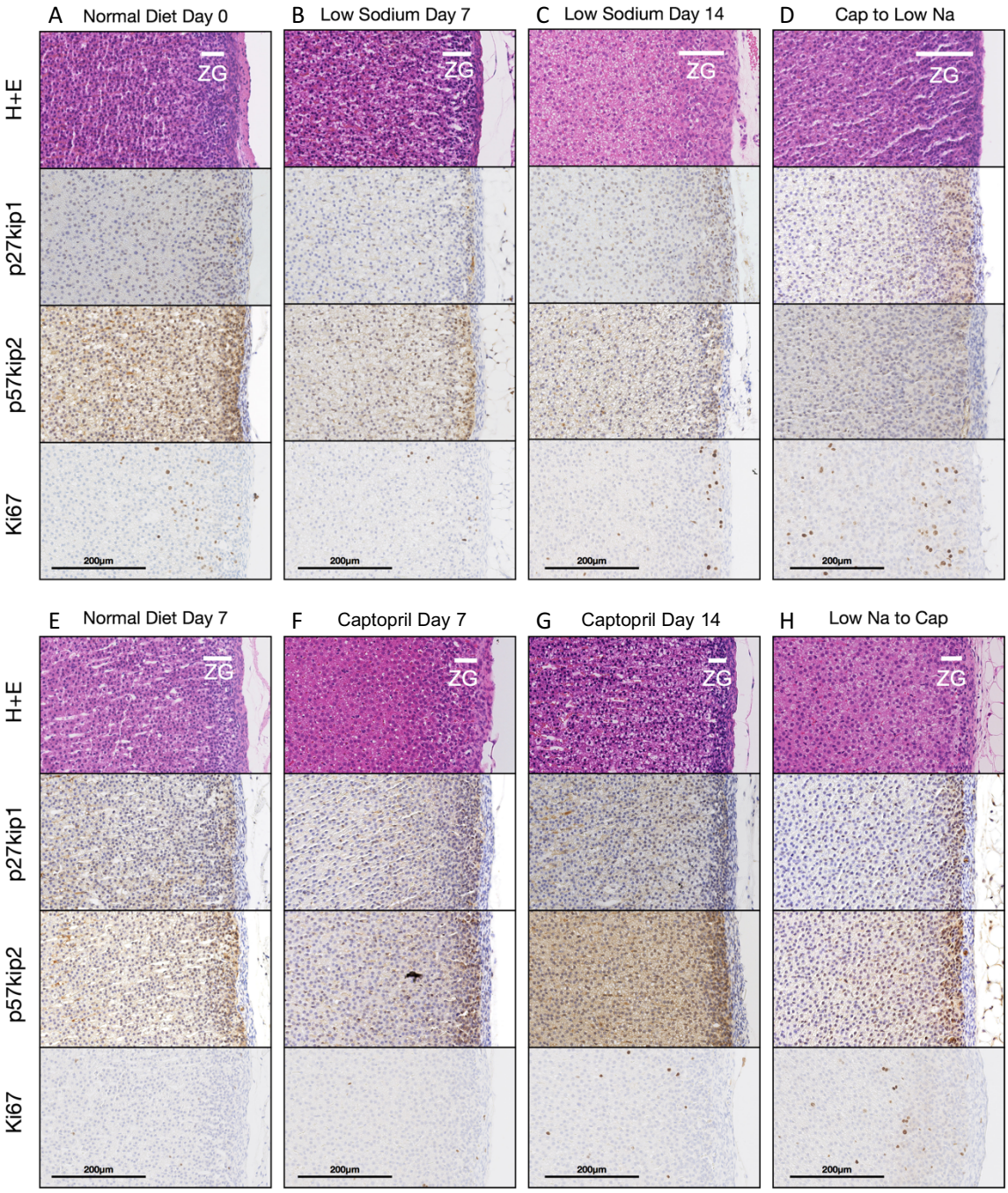

Figure 3.5 Immunostaining of p27, p57 and Ki67 under remodelling conditions
Rats were fed a normal diet (A and E), a low sodium diet (B-D), or a normal diet supplemented with captopril in their drinking water (F-H). Animals were sacrificed at 7 days and 14 days. At 7 days 3 rats from each experimental groups were crossed over to receive the reverse treatment (D and H). Representative images are shown. Sections were stained with H&E, and adjacent sections immunostained for p27kip1 (1:100), p57kip2 (1:100) and Ki67 (1:150). The number of Ki67 positive cells increases in the outer ZG after 7 days of low sodium diet (B), and is present throughout the ZG after 14 days (C), and in animals crossing over to low sodium diet from initial captopril treatment (D). Numbers of Ki67 positive cells are low in captopril treated animals (F-H). Nuclear expression of p27 and p57 is reduced in captopril treated animals (F-H) compared with those on low sodium diet (B-D), but p27 expression in the cytoplasm is apparent. Scale bars 200μm.

3.3.5 Activation of the RAS is associated with reduced ZG expression of p57, and cytoplasmic staining for p27.

Immunohistochemical staining for p27 and p57 is shown in figures 3.4 and 3.5 Nuclear p57 staining in the ZG was restricted to sub capsular cortex cells by placing rats on a low sodium diet for 7 days, with a further reduction in staining after 14 days. Inhibition of the RAS with captopril treatment increased the degree of nuclear p57 staining, with mild cytoplasmic staining in addition. Rats which were initially treated with captopril then switched to low sodium diet after 7 days also demonstrated reduced p57 staining. Animals initially on a low sodium diet who then received captopril demonstrated the strongest ZG p57 staining of all animals studied.

Activation of the RAS led to a reduction in nuclear p27 staining in the ZG, but the development of mild to moderate cytoplasmic staining. Cytoplasmic staining was mild and restricted to the immediate sub capsular cells after 7 days of low sodium diet, slightly expanded at 14 days, but most pronounced in the animals which had been converted to a low sodium diet after initial RAS inhibition with captopril. Captopril treated animals demonstrated an increase in nuclear p27 staining in the ZG that was present by 14 days, but was most prominent in animals initially on a low sodium diet.

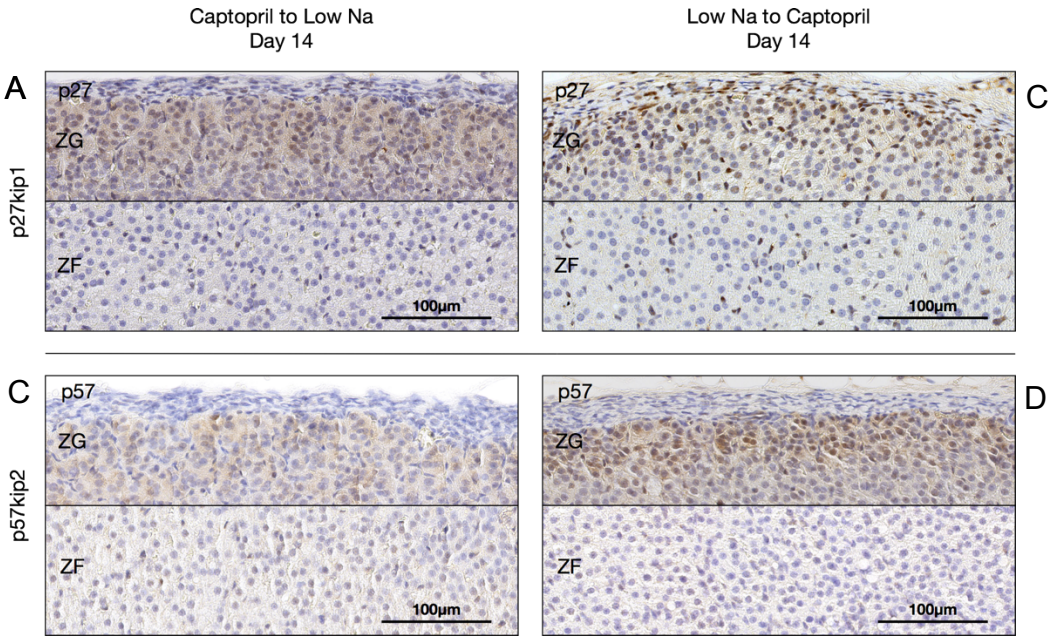

Figure 3.6 Higher magnification images of p27 and p57 staining in crossover arm.
3 animals from each experimental group (Low sodium diet; captopril) were switched to the opposite treatment after 7 days, and sacrificed at 14 days. Sections were immunostained for p27kip1 (1:100) and p57kip2 (1:100). Strong nuclear staining for both antibodies is present in

adrenals switched from low sodium diet to captopril treatment (B and D). Cytoplasmic staining of p27kip1 is present in the outer ZG from animals switched from captopril to low sodium diet (A)

3.4 Discussion

3.4.1 Lack of functional zona glomerulosa staining

CYP11B2, or p450 aldosterone synthase is the most appropriate functional marker of the zona glomerulosa. The lack of a commercially available antibody has hindered experimental investigation of this zone in the past. Antibodies to CYP11B2 in the rat have been available for some years, manufactured by the lab of Gomez-Sanchez *et al* (Engeland et al., 1996) and an aliquot was kindly provided. This gave successful staining on a few occasions, but unfortunately there was inconsistent staining of positive control sections. Colleagues within our own laboratory are attempting to establish a local supply of the antibody using the same method as Gomez Sanchez *et al* but this is not yet available. An alternative marker of the ZG was therefore sought.

In the rat adrenal cortex β-catenin is accumulated in a discrete peripheral domain which encompasses cells expressing CYP11B2 (ZG) but is distinct from cells that express Shh (ZU) (Drelon et al., 2015). Experimentally induced constitutive expression of β-catenin in the adrenal cortex of transgenic mice led to ectopic differentiation of ZG at the expense of ZF cells (Berthon et al., 2010), and it has been demonstrated that β-catenin controls the expression of the angiotensin receptor and CYP11B2 in both mouse and human adrenocortical cells (Berthon et al., 2014). Furthermore, WNT/β-catenin signalling has also been shown to specifically inhibit ZF differentiation (Walczak et al., 2014). Unfortunately, we failed to optimise staining with β-catenin, with significant positive staining even in negative controls. The angiotensin II receptor would be an additional marker that it would be informative to use to label the ZG.

Using functional markers to establish the location of the ZG is useful given the existence of the ZU between ZG and ZF in the rat which expresses neither CYP11B1 or CYP11B2, and is thought to be the location of Shh positive cells which signal to the stem cells in the capsule to differentiate, and are themselves candidate stem/progenitor cells (King, P. et al., 2009).

3.4.2 Increased proliferation within the ZG

RAS activation led to ZG expansion, and RAS inhibition led to its contraction. ZG cell hypertrophy, hyperplasia and increased expression of steroidogenic enzymes are expected when the RAS is stimulated (McEwan et al., 1996; McEwan et al., 1999). On H&E staining we were able to demonstrate that RAS activation led to ZG expansion, and RAS inhibition led to ZG contraction (Figure 3.5). In addition, there was hypertrophy within the cells of the outer ZG in rats on the RAS activating low sodium diet.

Activation of the RAS caused increased Ki67 immunopositivity in subcapsular cells at 7 days and throughout the ZG at 14 days. Animals switched from captopril to low sodium had the highest levels of Ki67 positivity in the ZG (Figure 3.3), but this was most prominent in the inner ZG at the boundary with the ZF (Figure 3.5 Panel D). There were scattered Ki67 positive cells within the ZF, particularly towards the inner ZF boundary in the captopril to low sodium cross over group (Figure 3.5 Panel H). This appearance was also apparent during the compensatory adrenal response to unilateral adrenalectomy in the rat (Engeland et al., 2005) but the authors do not comment on its significance. In the low-sodium to captopril cross over image shown (Fig 3.4), there is also a distinct pattern of staining for Ki67 at the ZF/ZR boundary. However, this is not consistent over the whole adrenal in that animal, nor is it present in adrenals from the other biological replicates. Furthermore, there was no statistical difference in ZF Ki67 positivity between any of the experimental groups (Fig 3.4), and this would be in keeping with previous reports of the effect of captopril treatment on rat adrenal ZF and ZR proliferation (McEwan et al., 1996).

It would be interesting to use additional markers of proliferation such as PCNA or phosphohistone H3 to establish whether this was a consistent finding. One possible explanation for this maybe that the Ki67 is labelling proliferating cells of the adrenal vasculature – aldosterone secreted from the ZG will be carried centrally through venous sinusoids, and could potentially stimulate proliferation and remodelling of the inner vascular plexus. Co-labelling of adrenal sections with Ki67 and CD31 to identify adrenal microvascular endothelial cells would clarify this possibility (Karaca et al., 2015).

It has been shown in the mouse that capsular cells delaminate and become steroidogenic (Kim et al., 2009), but centrifugal migration of BrdU labelled cells from the ZU into the

ZG in rats fed a low sodium diet has also been demonstrated (Mitani et al., 2003). Our data showing subcapsular Ki67 positivity after 7 days of RAS activation, spreading to the whole ZG at 14 days, along with cytoplasmic p27kip1 staining throughout the ZG would be consistent with centripetal migration. To determine if *bidirectional* migration was taking place it would be instructive to carry out lineage tracing experiments to follow the cells with cytoplasmic p27 localisation under conditions of RAS activation and ACTH stimulation.

3.4.3 p57 staining

Activation of the RAS reduced the expression of p57 in the ZG, while inhibition of the RAS increased expression. Kobayashi *et al* found that p57 expression was increased by ACTH treatment (Kobayashi et al., 2006) and therefore one may have expected the same pattern with activation of the RAS by low sodium diet. However, the level of p57 expression in control animals in their study was reported as zero, whereas previous studies had demonstrated significant cortical staining (Nagahama et al., 2001). Control animals in our study showed p57 staining throughout the cortex, with strongest staining in the subcapsular ZG (Figure 3.4 Panel C). This would be consistent with this as a location containing a stem or progenitor cell population.

Bilodeau *et al* suggested that the expression of p57 in the pituitary gland marked a cell population that was intermediate between progenitors and differentiated cells, and that p57 was itself a signal for differentiation. (Bilodeau et al., 2009). Differentiation in those cells was accompanied by loss of p57 expression and the appearance of p27. The high levels of p57 expression seen in the subcapsular cortex when the RAS system is inhibited, with loss of p57 when progenitor cells in the subcapsular adrenal cortex are stimulated to proliferate and differentiate would be predicted by this model. Downregulation of p27kip1 and p57kip2 would be predicted to release their inhibition of the Cyclin E-cdk2 complex, thereby facilitating the G1/S transition in the cell cycle, and allow these quiescent progenitor cells to proliferate (Musgrove, 2006). It will be necessary to perform dual immunostaining with p57 and Ki67 to confirm that cells which are p57-positive are not actually proliferating.

The role of p57 in adrenal pathology is also instructive here. In addition to adrenocortical hyperplasia in p57-/- mice (Zhang et al., 1997) there are two syndromes in human disease where changes in p57 affect adrenal development. Beckwith-Wiedemann syndrome (BWS) is the most common overgrowth syndrome: clinical findings include macrosomia, macroglossia, enlarged organs and hemihyperplasia. The syndrome is associated with a variety of adrenal tumours, and adrenocortical hyperplasia in the neonate (Mazzuco et al., 2012). The syndrome is associated with a number of genetic and epigenetic alterations related to the chromosome region 11p15.5, where the p57 gene CDKN1C is located. Mutations in CDKN1C are found in 10% of sporadic cases, and up to 40% of familial cases (Miozzo et al., 2014). Some of the mutations in BWS abrogate the CDK2-inhibitory ability of p57, while some affect sub cellular localisation and exclude it from the nucleus. There was a mild amount of cytoplasmic p57 staining in the ZG of low sodium treated rats in our study, suggesting that the sub cellular location of the protein may be important in normal physiology. As discussed below, it is clear that this is the case for p27.

In contrast, gain of function mutations in CDKN1C have been identified in patients with the IMAGe syndrome: intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia and genital anomalies (Vilain et al., 1999). Six missense mutations in the PCNA-binding domain in the C-terminal of CDKN1C have been reported, with evidence that this leads to increased stability and gain of function (Hamajima et al., 2013).

There is also frequent down-regulation of p57 in human cancers and this is correlated with aggressiveness in several of these (Guo et al., 2010). Down-regulation has been seen in adrenal tumours, although no CDKN1C mutations have yet been detected (Barzon et al., 2001).

3.4.4 p27 staining

Activation of the RAS in our study led to a reduction in p27 nuclear staining but an increase in cytoplasmic staining. There are reports in the literature of downregulation of p27 in rat adrenals treated with ACTH or other N-terminal POMC derived peptides (Kobayashi et al., 2006; de Mendonca et al., 2013). Kobayashi *et al* showed a transient inhibition of p27 expression within the whole adrenal by western blot assay of the protein, and immunohistochemistry. De Mendonca *et al* reported reduced p27 by both methods,

but representative figures of p27 immunohistochemistry in their paper clearly demonstrate increased cytoplasmic staining in the ZG of rats treated with ACTH and N-POC 1-29. Interestingly, chronic ACTH treatment led first to a reduction in p27 and later an increase in the Kobayashi *et al* study, measured at time points up to 8 days.

The p27 $^{-/-}$ mouse has medullary hyperplasia, consistent with the high expression of p27 in this region, but no reported cortical defects (Nakayama et al., 1996). These mice develop adenomas of the intermediate lobe of the pituitary gland, and a novel human multiple endocrine neoplasia syndrome - MEN4 - has been described in which there are heterozygous mutations in p27 (Marinoni & Pellegata, 2011). The tumour suppressor role of p27 suggested by this phenotype is supported by the low amounts of p27 found in a number of human malignancies, including breast, colon, prostate and lung cancer. It is associated - like p57 - with an increase in tumour aggressiveness (Besson et al., 2008)

Other malignancies show mislocalisation of p27 to the cytoplasm: this occurs in up to 40% of breast cancer (Viglietto et al., 2002); it is also a negative prognostic factor in these cancers (Tanaka, T. & Iino, M., 2014). Additionally, whereas the $p27^{-/-}$ mouse develops only pituitary tumours, the p27CK- mouse which contains a mutant protein that lacks the ability to interact with CDKs and cyclins, develops hyperplasia and tumours of multiple organs (Besson et al., 2007). Lung tumours in this model arise from a population of bronchoalveolar stem cells, supporting the hypothesis that p27 is a stem cell marker. This is supported by the observation that both p27 and p57 have a role in maintaining quiescence in haematopoietic stem cells (Zou et al., 2011). Interestingly, in this tissue a deficiency of p57 leads to up regulation of p27, suggesting a degree of redundancy in the system which was also proposed by Bilodeau et al in the mouse pituitary (Bilodeau et al., 2009).

3.4.5 Cell migration

The effect of cytoplasmic p27 in malignancy, and behaviour of the $p27^{CK-}$ mouse suggests an oncogenic effect of p27 in addition to its tumour suppressor role. This may lie in its role in modulation of cell migration. p27 promotes the migration of metastatic hepatocellular carcinoma cells *in vitro*, via a reduction in the activity of the RhoA GTPase (Nagahara et al., 1998; Wang, X. Q. et al., 2008). The Rho GTPases are a group of GTP/GDP binding proteins that are part of the Ras superfamily of GTP-binding

proteins(Ridley, 2013). They are well known for their roles in cell migration, though effects on actin and microtubule dynamics, adhesion between individual cells and between cells and the extracellular matrix (ECM) (Parsons et al., 2010). RhoA-GTP activates Rho-kinases ROCK1 and ROCK2; inhibition of this pathway by p27 leads to a decrease in focal adhesions, leading to an increase in cell motility (Larrea et al., 2009).

Regulation of RhoA by p27 is critical for proper migration of neural progenitor cells(Nguyen et al., 2006), while cytoplasmic p27 is required for *in vivo* infiltration of macrophages into tumours in mice(Gui et al., 2014). Although most of the p27 effects on cell migration are to encourage it, this seems to be dependent on the precise cellular context. Progesterone inhibits the migration of rat aorta smooth muscle cells by upregulating p27, while estradiol reduces p27 and enhances proliferation and migration of vascular endothelial cells (Wang, H.-C. & Lee, 2014; Oviedo et al., 2011). This may reflect the particular mode of migration - cells lacking in p27 have been shown to migrate in an amoeboid fashion rather than a mesenchymal one (Belletti et al., 2010). The effect of p27 on cellular migration is modulated by protein phosphorylation at various sites. For instance, phosphorylation at Ser10 target it for export to the cytoplasm, while binding to RhoA is facilitated by phosphorylation at Thr198 (Larrea et al., 2009).

3.4.6 Stem cells and migration

We observed that RAS activation reduced nuclear immunostaining for p27 in the ZG, but increased cytoplasmic staining. On the basis of these results, a model can be proposed that under conditions of RAS activation there is down regulation of nuclear p27 and p57 in the ZG, allowing progenitor cells to proliferate and differentiate into steroidogenic cells. Downregulation of p27 and p57 allows the cyclin E-cdk2 complex to phosphorylate p27, enabling its translocation to the cytoplasm (Sheaff et al., 1997). An accumulation of cytoplasmic p27 provides a mechanism whereby cell migration may be induced, mediated by the inhibition of the RhoA GTPase.

3.4.7 Further Experiments

Optimisation of immunostaining for CYP11B2 is essential to further elucidate the mechanisms by which alterations in p27 and p57 are involved in mediating the effects of RAS activation. The change in nuclear staining of p27 and p57 is grossly similar, but seems to affect different cell populations, p27 is mainly in the capsule and subjacent

cortex, whereas p57 is expressed throughout the ZG. Characterising these cells in more detail will require double immunostaining with B2. Double immunostaining is more straightforward using immunofluorescence, although this technique can be less successful in FFPE sections due to background fluorescence induced by the fixation process. For each rat the contralateral adrenal gland was harvested in liquid nitrogen and can therefore be used for immunofluorescent staining. The assertion that downregulation of p57 is enabling proliferation must also be tested with dual immuno-staining of p57 and Ki67 – if cells are both p57 and Ki67 positive then this would have to be rejected or at least substantially modified. Similar dual staining for p27 and Ki67 is also necessary for the same reasons.

Determining the specific time points and locations at which p27 and p57 are expressed during differentiation could be effectively studied using a lineage tracing approach. King *et al* defined the Shh lineage (ZU) and Gli1 lineage (inner capsule) using the *cre* driver alleles *Shh-creT2* and *Gli-creT2,* showing that the Gli1 expressing cells are the progenitors of steroidogenic cells (King, P. et al., 2009). Following the expression of p27 and p57 in both of these models following tamoxifen treatment would help to identify if they have separate roles in this process. Performing the immunostaining at multiple shorter intervals would also be informative however, although activation of the RAS system using a low sodium diet would be unsuitable. Infusion of rats with angiotensin II subcutaneously via osmotic minipump (Ruiz-Ortega et al., 2001) and performing the same immunostaining over a period of 24-48 hours may be a more appropriate method.

The mechanism by which cytoplasmic p27 is proposed to enable cell migration of newly differentiating cells can be further investigated by immunostaining for the phosphorylated forms of p27 which localise it to the cytoplasm, and immunostaining for RhoA-GTPase, which would be expected to be downregulated in RAS activation. Expression of p27 and p57 in dispersed rat adrenal cells treated with ACTH or AngII could be performed by quantitative PCR or western blot for the protein. Cell migration assays in such an *in vitro* system are unlikely to be particularly informative, although cells lacking p27 tend to have a more amoeboid mode of migration, which is the most straightforward to investigate *in vitro.* It would also be instructive to observe the response of the p27-/- mouse adrenal to stimulation of the RAS and activation of the HPA axis, especially with regard to changes

in p57, and to see whether p57 behaves more like p27 in this model, given suggestions that there is some degree of redundancy between them.

3.4.8 Conclusion

These experiments have demonstrated RAS activation leading to the histological appearance of ZG expansion in the rat adrenal as defined by H+E staining, with Ki67 immunostaining showing early subcapsular proliferation, and later proliferation at the ZG/ZF interface. Nuclear expression of the cyclin-dependent kinase inhibitors p27kip1 and p57kip2 is suppressed when the RAS system is activated, and upregulated in slightly different locations when it is inhibited, suggesting that there may be some overlap in their functions. Cytoplasmic p27 expression is increased by RAS activation, and this is known to have a role in enabling cell migration in other systems, and so may be involved in enabling migration of newly differentiated steroidogenic cells within the ZG.

Chapter 4 A case of ACTH-independent Cushing's syndrome

4.1 Aims

This chapter describes the case of a patient with ACTH-independent Cushing's syndrome and a gastro-intestinal stromal tumour (GIST). It aims to demonstrate that the GIST is directly responsible for stimulation of cortisol production from the adrenals.

4.2 Introduction

Cushing's syndrome is the result of chronic overexposure to glucocorticoid, either from oral, topical or inhaled corticosteroid, or endogenous overproduction of cortisol from the adrenal gland. Endogenous Cushing's syndrome is most commonly ACTH-dependent. When it is the result of dysregulated secretion of ACTH from a corticotroph tumour of the pituitary gland it is termed Cushing's disease.

The ectopic ACTH syndrome (EAS) represents 20% of ACTH dependent Cushing's syndrome(Newell-Price et al., 2006). Neuroendocrine tumours secreting ACTH are most commonly found in the lung (bronchial carcinoids, small cell lung cancer) or the GI tract and pancreas. Phaeochromocytomas and medullary cell thyroid cancers also make a significant contribution, but in up to 38% of cases no tumour is identified (Alexandraki & Grossman, 2010). Ectopic secretion of CRH from neuroendocrine tumours is well recognised but extremely rare, and may occur with co-secretion of ACTH (Carey et al., 1984). A host of non-neuroendocrine tumours including breast cancer and melanoma have been identified in isolated case-reports as the source of ectopic ACTH secretion, leading Alexandraki *et al* to conclude that almost any tumour may be associated with EAS (Alexandraki & Grossman, 2010).

Around 20-30% of Cushing's syndrome is ACTH-independent (Lacroix et al., 2015). This is usually the result of a single cortisol-producing adrenal adenoma, but may be due to bilateral adrenal hyperplasia. ACTH-independent macronodular adrenal hyperplasia (AIMAH) may be caused by ectopic expression of G-protein linked receptors for ligands other than ACTH on the surface of adrenocortical cells (Lacroix et al., 2001), or a

mutation in the armadillo containing repeat 5 gene (Assie et al., 2013). Primary pigmented nodular adrenal disease (PPNAD) is a form of ACTH-independent Cushing's usually diagnosed in childhood, and most often as part of the Carney complex. It is the result of inactivating mutations of the regulatory subunit type 1a of PKA (PRKAR1A) (Kirschner et al., 2000), or in the phosphodiesterase enzymes PDE11A or PDE8B (Horvath, A. et al., 2006), leading to up regulation of PKA mediated signalling pathways downstream of the MC2R.

Gastrointestinal stromal tumours are mesenchymal tumours of the GI tract which are believed to arise from the gut's pacemaker cells - the interstitial cells of Cajal (Corless et al., 2004). Sporadically occurring GISTs are characterised by activating mutations in KIT, PDGFRA or BRAF (Yang et al., 2008), while there are a host of multiple tumour syndromes which have GISTs as a major feature (Burgoyne et al., 2014). Of these syndromes, a number include synchronous or metachronous endocrine tumours, but the capacity for endocrine function in GISTs themselves is restricted to case reports of paraneoplastic hypoglycemia from IGF-II production (Beckers et al., 2003) and in vitro studies which identified some molecular characteristics which GISTs share with endocrine tissues (Ekeblad et al., 2006; Bumming et al., 2007).

4.3 Case History

4.3.1 Clinical Assessment

A 51-year-old lady with severe hypertension developed proximal myopathy with atrophic and hyperpigmented skin, and weight gain of 15kg over a 9-month period. She had suffered from a low impact fracture of the third metatarsal in the previous year which was managed conservatively, but prompted an assessment of bone density, and a subsequent diagnosis of osteoporosis. Her past medical history was remarkable for a bilateral oophorectomy 2 years previously. There was no family history of any endocrine disease. She had smoked 20 cigarettes daily for 30 years, but had stopped a year before.

She had achieved reasonable control of her blood pressure with spironolactone 25mg daily, lisinopril 10mg daily and indapamide 250mg daily. She used a topical oestrogen

preparation to prevent vasomotor symptoms of the menopause, and once weekly alendronic acid for osteoporosis.

On clinical examination she was profoundly cushingoid (Figure 4.1), with a body mass index of 37kg/m^2. Blood pressure was 120/75 mmHg with no postural drop. She had developed a tanned appearance over her face and the sun-exposed areas of her upper chest; she was red-haired and had never previously tanned in this way. A diagnosis of Cushing's syndrome was suspected based on these clinical findings.

Figure 4.1 Clinical Photography
Typical appearances of Cushing's syndrome are apparent with centripetal obesity, midscapular fat pad and muscle wasting of the arms and legs. The patient is profoundly pigmented in her face and upper chest.

Routine haematological tests revealed evidence of an iron deficiency anaemia: Hb 11.1 g/dl (10.9-14.5), MCV 79.9fl (83-98), serum iron 5µmol/l (9-30). Screening endocrine biochemistry results are demonstrated in tables 4.1-4.2. Further biochemical assessment demonstrated complete loss of circadian rhythm of cortisol secretion and failure of suppression of cortisol during a standard low dose dexamethasone suppression test (LDDST) (2mg/day for 48 hours; cortisol levels 761nmol at baseline, 771 nmol/l at 48h. (Table 4.3, 4.4)). Levels of adrenocorticotrophic hormone (ACTH) were consistently undetectable. Serum cortisol levels did not alter in response to subcutaneous octreotide

or a mixed meal tolerance test (Tables 4.5, 4.6). A CRH test demonstrated no change in serum cortisol or ACTH (Table 4.7). Fasting gut peptide levels were within normal limits (Table 4.8)

Table 4.1 - Biochemistry

Sodium	141	135-145 mmol/l
Potassium	4.3	3.5-5.1 mmol/l
Chloride	104	98-106 mmol/l
Bicarbonate	31	22-29 mmol/l
Urea	6.8	2.5-6.4 mmol/l
Creatinine	64	44-80 µmol/l
Bilirubin	4	1-17 µmol/l
ALT	34	7-35 IU/l
ALP	63	35-104 IU/l
Protein	62	60-83 g/l
Albumin	37	35-50 g/l
Corr Calcium	2.39	2.15-2.65 mmol/l
Phosphate	1.14	0.8-1.5 mmol/l
Magnesium	0.86	0.7-1.0 mmol/l

Table 4.2 - Endocrine Biochemistry

		Reference Range
Cortisol	770	200-600 nmol/l
Androstenedione	9.9	2.0-5.4 nmol/l
DHEA	8.5	1.6-7.8 nmol/l
Testosterone	1.4	< 3.0 nmol/l
ACTH	< 5	<50 ng/
free T4	13.4	10.5-24.5 pmol/l
TSH	0.44	0.3-4.0 mU/l
LH	< 0.3	4-14 U/l
FSH	2.8	1.5-8 U/l
Oestradiol	244	250-1000 pmol/l
Prolactin	423	< 496 mU/l

Table 4.3 - Cortisol Day Curve

Time	ACTH ng/l	Cortisol nmol/l
900	< 5	681
1200	< 5	751
1500		608
1800		696
2100		658

Table 4.4 - Low dose dexamethasone suppression test

Time/h	Cortisol nmol/l
0	761
24	737
48	771

Table 4.5 - Octreotide Test

Time / hr	Cortisol nmol/l
0	569
1	547
2	548
3	495
4	518
5	526
6	482
7	502
8	530

Table 4.6 - Mixed Meal Test

Time / min	Cortisol nmol/l
0	623
15	610
30	629
45	630
60	584
90	588
120	604

Table 4.7 - CRH test

Time min	ACTH ng/l	Cortisol nmol/l
-30	<5	604
-15	<5	608
0	<5	602
15	<5	580
30	<5	587
45	<5	546
60	<5	543
90	<5	585
120	<5	573

Table 4.8 - Fasting gut peptides

	Time 0h	Time 4h	Reference Range
Chromogranin A	50	44	< 60 pmol/l
Chromogranin B	77	79	< 150 pmol/l
Gastrin	150	125	< 40 pmol/l
Glucagon	17	6	< 50 pmol/l
Pancreatic polypeptide	42	15	< 300 pmol/l
Somatostatin	32	50	< 150 pmol/l
VIP	5	6	< 30 pmol/l

Table 4.9 - Hydrocortisone Day Curve (2 years post op)

Time	HC Dose	ACTH ng/l	Cortisol nmol/l
0	10mg	8	95
30m			187
1h			506
2h			531
3h			336
5h	5mg		189
7h			311
9h			429
10h			290

Dual emission X-ray absorptiometry (DEXA) confirmed reduced bone mineral density (BMD) at the lumbar spine and femoral neck (T scores -2.2 and -1.4 respectively; measured in SD from the mean BMD in a healthy population)

Cross-sectional imaging revealed bilateral nodular enlargement of the adrenal glands (right 5.5 x 4.9cm; left 1.7 x 3cm plus smaller nodules). The right gland contained areas of high and low density, with contrast washout of 73%. The nodules on the left all measured less than 10HU pre-contrast, in keeping with lipid-rich adenomata. In addition to these, there was a 4.1cm mass lesion consistent with a gastrointestinal stromal tumour (GIST), arising exophytically from the greater curvature of the stomach (Figure 4.2).

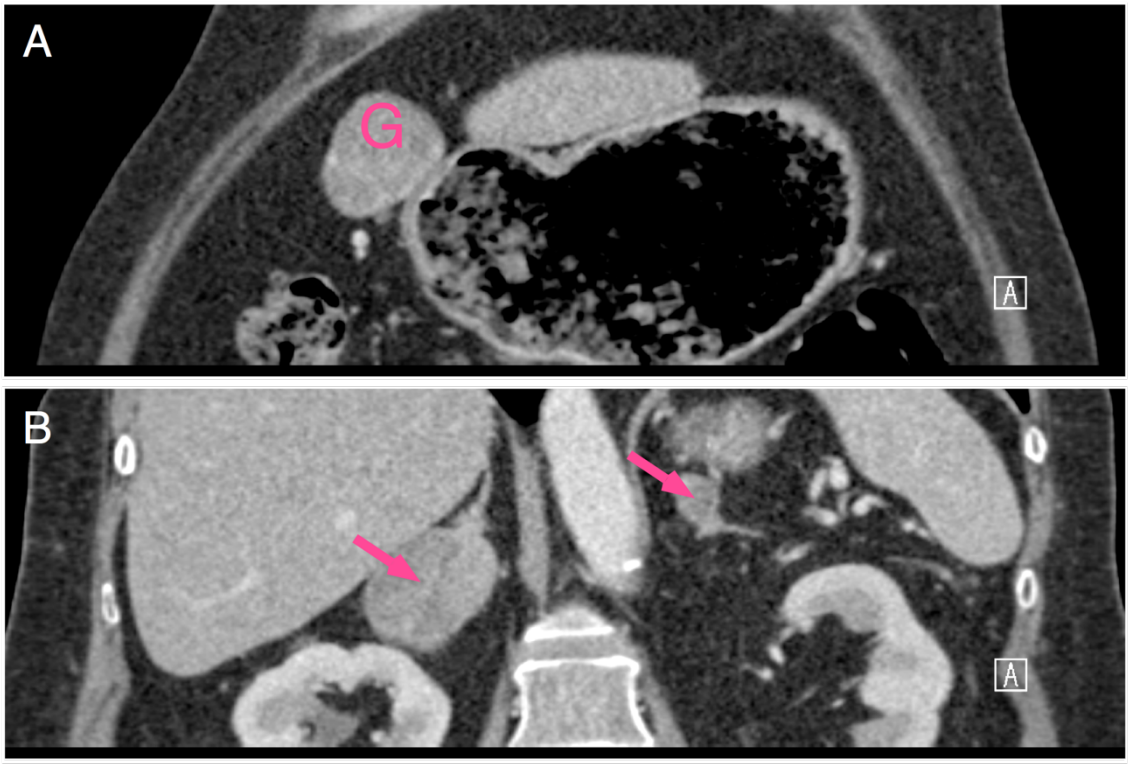

Figure 4.2 Non-contrast CT scan of the abdomen
Coronal unenhanced CT scans of the abdomen show an exophytic lesion (G) arising from the stomach in the upper panel, consistent with a GIST. Bilateral macronodular adrenal hyperplasia is seen in the lower panel (arrows), larger on the right than the left.

A hypothesis was formed that the GIST was secreting some factor with the capacity for stimulating adrenal growth and cortisol secretion. For this reason, and although there was no previous evidence in the literature for GIST hormone secretion leading to Cushing's syndrome, consideration was given to performing a surgical procedure to remove the GIST alone. The large size of the right adrenal gland raised radiological

concern at the possibility of malignancy, so a final surgical decision was taken to remove the GIST and the right adrenal in a combined procedure. Surgery was carried out after several weeks of treatment with oral metyrapone. The pathological specimens are shown in figure 4.3.

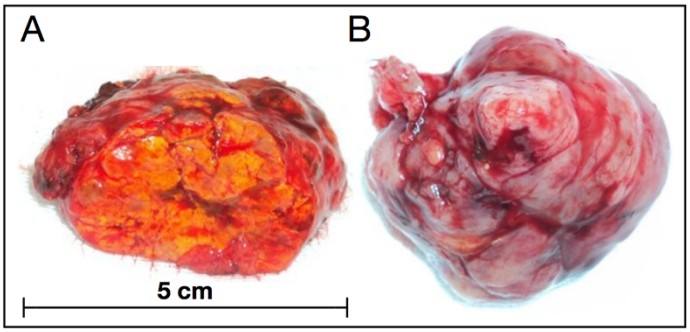

Figure 4.3 Surgical specimens of right adrenal gland and GIST

4.3.2 Histological Examination

The right adrenal gland weighed 36.5g and measured 48 x 43 x 25mm, with multiple pigmented nodules, the largest of which of 21 x 15 x 34mm. On microscopy the glad contained poorly defined nodules of densely packed large cells with eosinophilic cytoplasm and markedly pleomorphic nuclei with prominent nucleoli (Fig 4.4). Most of the large cells contained pigment. No mitotic figures were seen in 50 high power fields which were examined. The nodules themselves were unencapsulated, and contained adipose tissue and scattered lymphoid aggregates. The degree of pigmentation led to a concern from the examining pathologist that primary pigmented nodular adrenal disease (PPNAD) ought to be excluded, with the caveat that it would be rare in this age group. As described in more detail in Chapter 5, PPNAD is characterised by a pigmented and micro-nodular adrenal, with Cushing's syndrome usually developing in childhood (Horvath, A. & Stratakis, 2007) - neither the histological appearance of the adrenal, nor the clinical history would be in keeping with this disease.

The gastric tumour measured 45 x 49 x 30mm with a thin fibrous stalk of 5mm in length. Extensive sampling demonstrated the lesion to be moderately cellular and composes of sheets of epithelioid cells with round vesicular nuclei, inconspicuous basophilic nucleoli and moderate amounts of eosinophilic cytoplasm. The lesion was fairly vascular with some areas of haemorrhagic infarction. There was a mild, patchy lymphocytic infiltrate and mitotic figures at 2 per 50 high power fields. Immunohistochemistry demonstrated strong and diffusely positive staining for DOG-1 and focal but unequivocal cytoplasmic

and membrane positivity for CD-117 (c-kit) (Figure 4.4). Tumour cells were negative for CD34, desmin, smooth-muscle actin (SMA), S100 and AE1/3. The Ki67 proliferative index was 15%. The overall morphological and immunohistochemical profile of the tumour confirmed its identity as a GIST. Of particular note, immunostaining for ACTH was negative. The GIST was classified as “Low Risk” according to the National Institute for Health (NIH) classification.

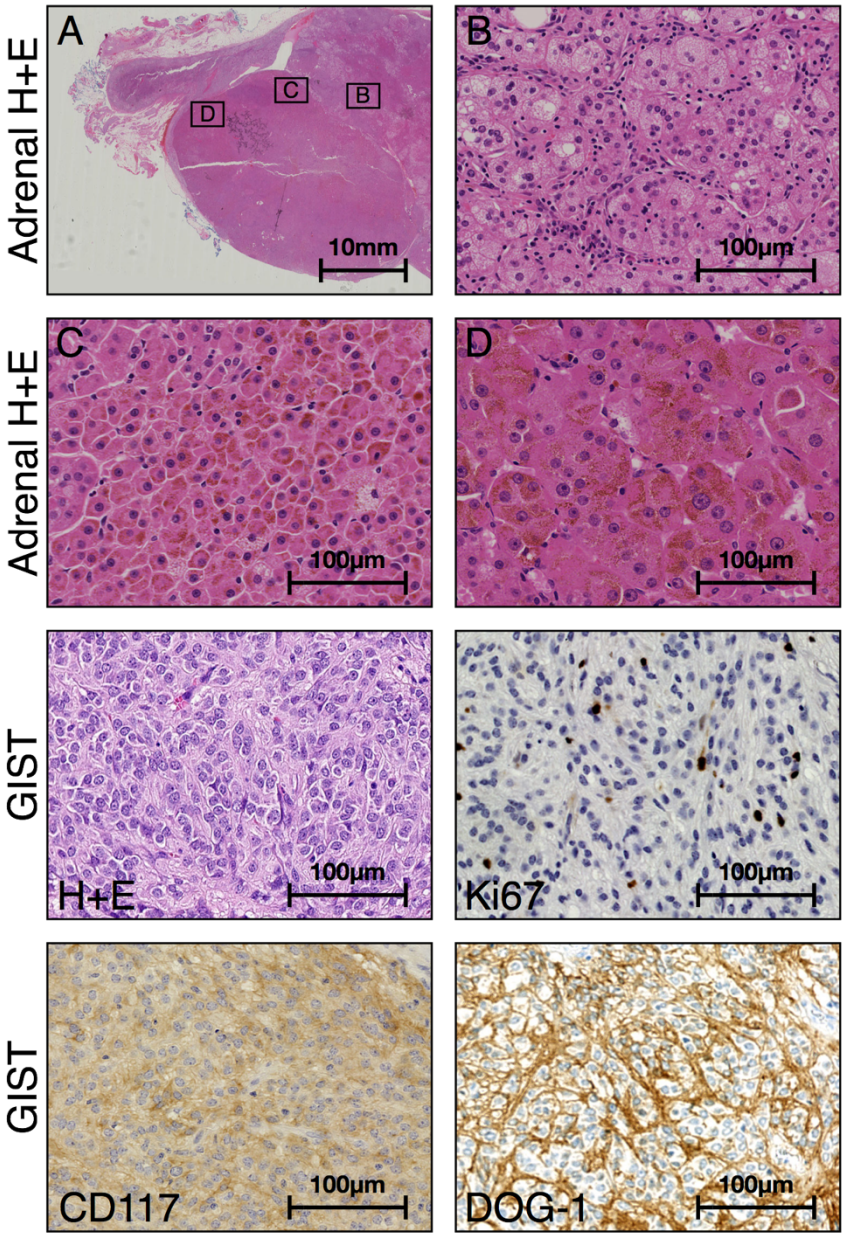

Figure 4.4. Histological specimens of adrenal gland and GIST.
The adrenal gland contained multiple large nodules with a pleomorphic appearance, and heavy pigmentation (A-D). Positive immunostaining with CD117 and DOG-1 antibodies confirmed that

the gastric tumour was a GIST. The Ki67 proliferation index was 15% but the GIST was classed as low risk according to NIH criteria. Staining was carried out according to methods (Section 2.5)

4.3.3 Clinical outcome

Post-operatively, 9am serum cortisol was 49 nmol/l and she commenced oral hydrocortisone replacement. She remains in remission from Cushing's syndrome three years on from surgery, still dependent on supplementary hydrocortisone. Surveillance imaging of the abdomen has demonstrated a slight reduction in the size of the remaining left adrenal gland (Figure 4.5). There has been no recurrence of the GIST and no use of chemotherapy or radiotherapy.

Interestingly, the patient developed a seronegative arthropathy in the first year following her surgery, and required high dose glucocorticoid treatment. When stabilised on methotrexate, her prednisolone dose was weaned down and she was switched to oral hydrocortisone, 10mg on waking and 5 mg early afternoon. A hydrocortisone day curve was performed 2 years after surgery (Table 4.9) and demonstrates low baseline ACTH levels, and a low pre-dose cortisol level of 95nmol/l. She remains on oral hydrocortisone at the time of writing, although this has been reduced to 5mg twice daily.

Her skin pigmentation entirely resolved within 3 months of surgery, and has not recurred.

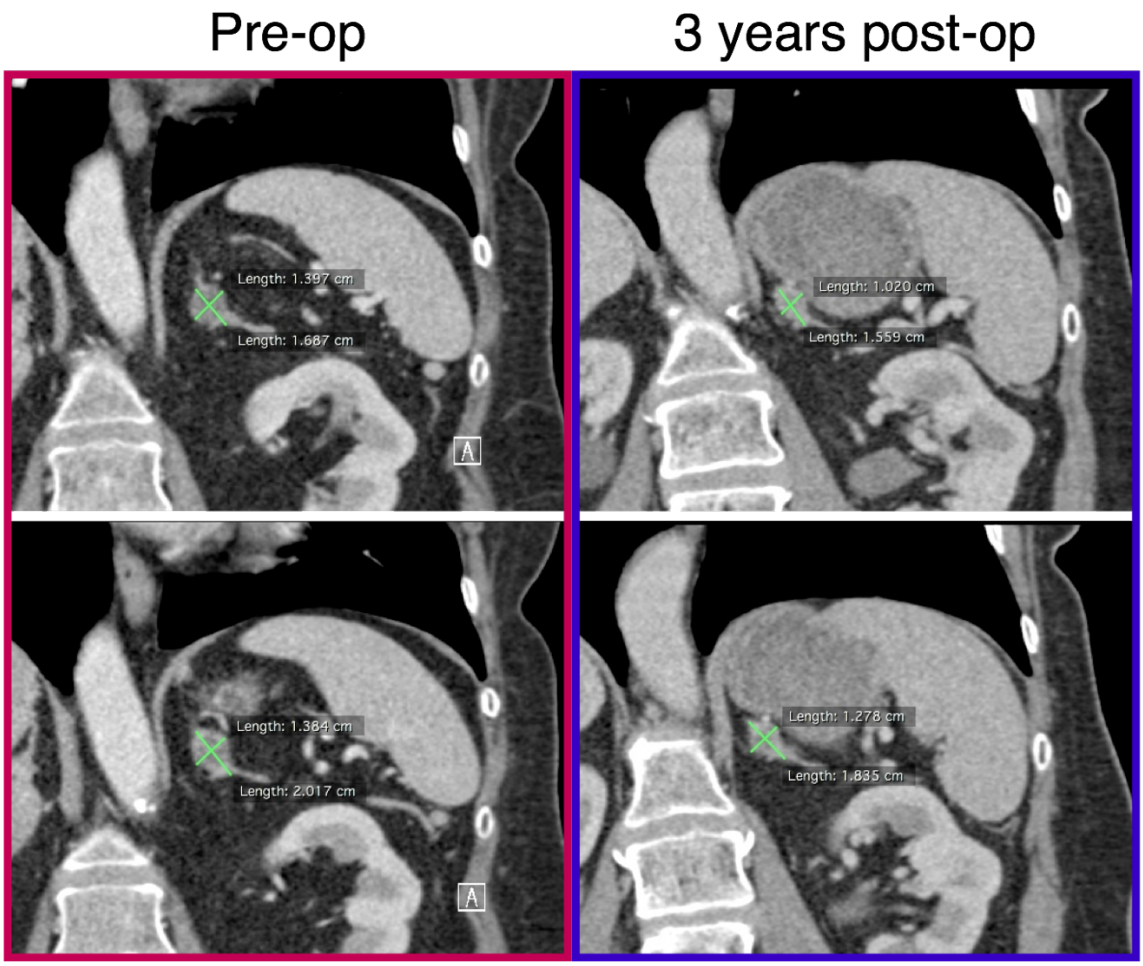

Figure 4.5. Pre and post-op portal venous phase CT images of the abdomen
Re-imaging of the left adrenal gland 3 years post-operatively demonstrated a reduction in size. Upper and lower panels show equivalent slices. Left adrenal size: Upper images 1.397 x 1.687cm to 1.020 x 1.559 cm; lower images: 1.384 x 2.017cm to 1.278 x 1.835cm.

4.3.4 Negative control GIST

A GIST was acquired from a 72-year-old man with no significant past medical history, and no clinical or biochemical evidence of Cushing's syndrome. The tumour in this case was also taken from the stomach wall, and measured 35 x 12 x 25mm. It had a more variable appearance on microscopy, with some areas composed of spindle cells arranged in fascicles, and more epithelioid cells in others. Immunohistochemistry showed positive staining for CD117, DOG-1 and CD34, with negative staining for S100 and desmin (Figure 4.6). The tumour was completely excised and rated as "Low Risk" according to the NIH classification.

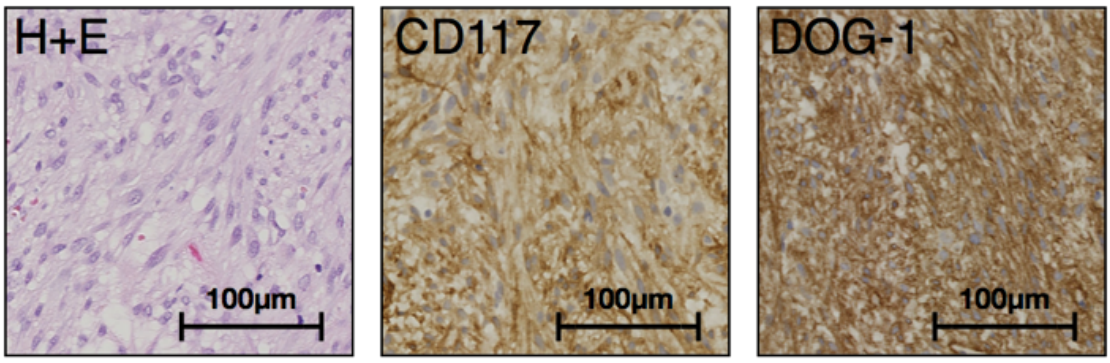

Figure 4.6. Immunohistochemical images of negative control GIST
A GIST from a patient with no clinical or biochemical evidence of Cushing's syndrome was used as a negative control. H+E staining demonstrates spindle cells arranged in fascicles. Immunostaining was carried out with antibodies against CD117 (1:1000) and DOG-1 (1:300) according to methods laid out in Section 2.5, Table 2.1. Immunostaining was positive for both antibodies, confirming that this tumour was a GIST

4.4 Experimental Results

4.4.1 GIST expresses a somatic mutation in PDGFRA

Sanger sequencing of genomic DNA from the GIST tumour revealed a previously reported heterozygous PDGFRA mutation c.2525A>T (pAsp842Val) that was not present in leucocyte DNA (Figure 4.7). No mutations were detected in KIT, BRAF or SDHB. The majority of GIST mutations are heterozygous, and pAsp842Val is the most common mutation in GISTs which do not have a KIT mutation (Xu, C.-W. et al., 2015). Further confirmation of the lack of SDHB mutation was provided by a normal pattern of SHDB immunohistochemical staining (Figure 4.7).

Sequencing of genomic DNA from the patient's leucocytes and the excised right adrenal gland failed to demonstrate a mutation in PRKAR1A, PDE11A or ARMC5.

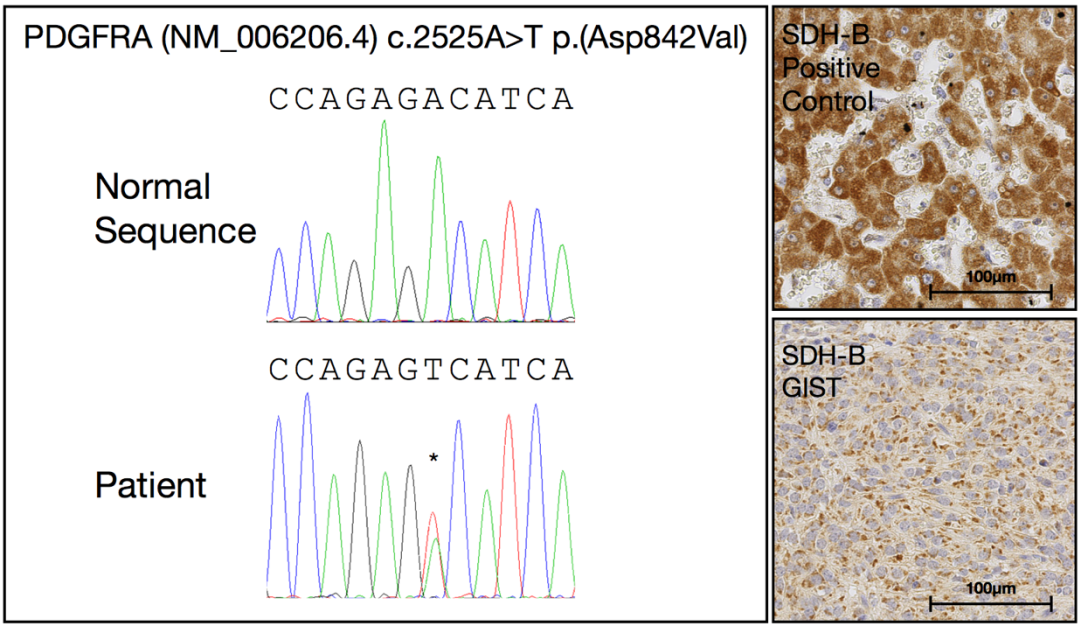

Figure 4.7 GIST carries PDGFRA mutation pAsp842Val
Sanger sequencing electropherogram on the left shows a heterozygous PDGFRA mutation c.2525A>T (pAsp842Val) that was not present in leucocyte DNA. On the left normal SDH-B staining is demonstrated, effectively ruling out a mutation in any of the SDH chain enzymes A-D.

4.4.2 Supernatant from GIST primary culture stimulates cortisol release in vitro

Primary cultures were successfully established from the GIST tumour of the patient with Cushing's syndrome (GC) and patient without Cushing's syndrome (NC). Both cultures demonstrated positive immunocytochemical staining with the GIST markers CD117 and DOG-1 (Figure 4.8).

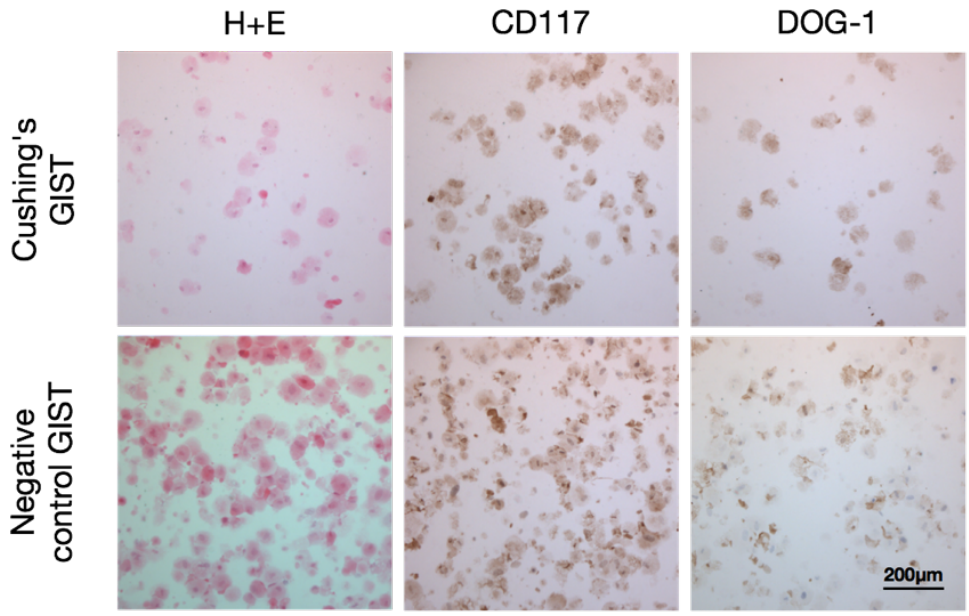

Figure 4.8 Immunocytochemistry of cultured GIST cells
Cultured GIST cells were trypsinised and resuspended, then centrifuged at 800rpm for 5 minutes to deposit them on to standard glass slides for immunostaining. Immunochemistry was carried out according to methods laid out in section 2.6.3. Cells from both GIST primary cultures stained positively for the GIST markers CD117 and DOG-1.

Supernatant from each primary culture was assayed and found to contain no significant trace of cortisol or ACTH. Supernatant from the GIST culture (GCM) induced a significant elevation in cortisol production from H295R cells (Figure 4.9 panel A). There was no significant elevation in cortisol when cells were incubated with supernatant from the negative control GIST culture (NCM). This rise was prevented by co-incubation with the PKA pathway inhibitor H89, and by pre-heating the media to 65ºC to denature protein (Figure 4.9 A). Culture supernatant from the first GIST passages led to cortisol production in excess of that produced by the adenylyl cyclase activator forskolin. This effect fell with later passages as can be seen in figure 4.19 (Panel A): supernatant from the third passage of GIST primary culture causes less H295R cortisol production than forskolin, whereas supernatant from the first passage (Figure 4.9 Panel A) causes more cortisol production than forskolin.

In addition to H295R cells, dispersed rat adrenal cells were also incubated with supernatants from both GIST cultures. These cells are not capable of producing cortisol, but GC supernatant led to a significant increase in corticosterone production (Figure 4.9 panel b).

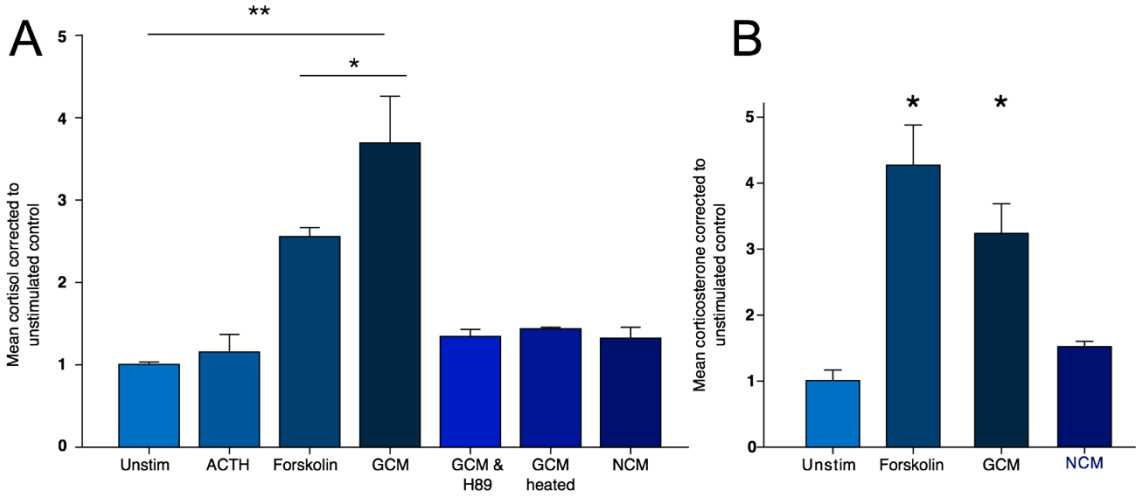

Figure 4.9 GIST-supernatant induced cortisol and corticosterone secretion

(A) H295R cells were incubated overnight with GIST culture medium (GCM), then media removed and assayed for cortisol. GCM induced a rise in cortisol secretion that was significantly greater than the positive control treatment with the cAMP signalling activator forskolin. This rise was abolished by co-incubation with the PKA pathway inhibitor H89, or by pre-heating the media to denature protein. Conditioned media from the negative control GIST (NCM) did not stimulate cortisol secretion.

(B) Dispersed rat adrenal cells were incubated overnight with GCM and NCM as above, and media analysed for corticosterone production by ELISA. GCM induced a statistically significant rise in corticosterone secretion above control. All experiments performed in triplicate. ** p< 0.01; *p< 0.05

Quantitative-PCR analysis of RNA extracted from H295R cells incubated with GIST culture supernatant demonstrated increased expression of the steroidogenic pathway components SF-1 and StAR. (Figure 4.10).

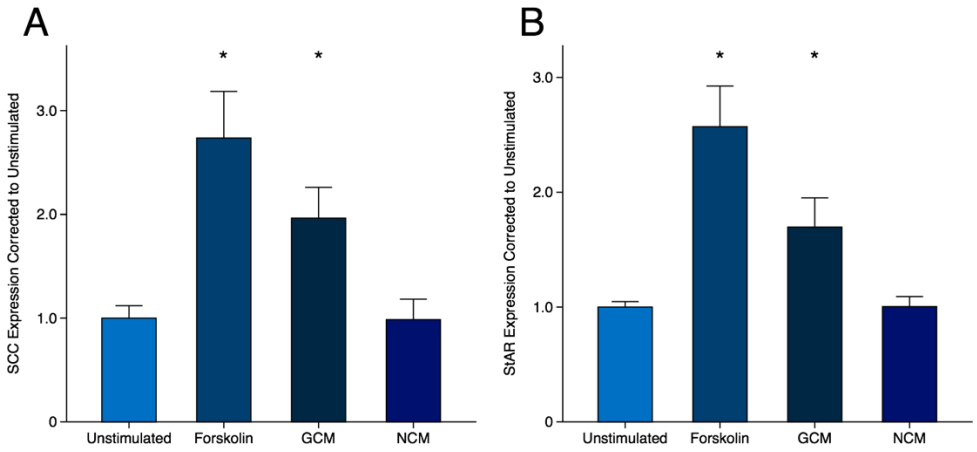

Figure 4.10 GCM upregulates the steroidogenic enzymes StAR and SCC

cDNA was harvested from H295R cells following an overnight incubation with GIST-conditioned medium (GCM), and demonstrated increased expression of steroidogenic acute regulatory protein (StAR) and side chain cleavage enzyme (SCC) by quantitative RT-PCR. There was no such increase with supernatant from the control GIST culture (NCM). All experiments in triplicate. * p<0.05

4.4.3 GIST culture supernatant does not interfere with the ACTH assay.

The absence of ACTH on electro-chemiluminescent assay of GIST culture supernatant, and from patient GC's serum, was considered to be the possible consequence of a factor which was interfering with the assay. A serum sample of ACTH taken from a patient with Addison's disease prior to their morning dose of oral hydrocortisone was used as a possible control. The addition of GC supernatant in various proportions made no significant difference to the reported ACTH content of this serum sample (Figure 4.11)

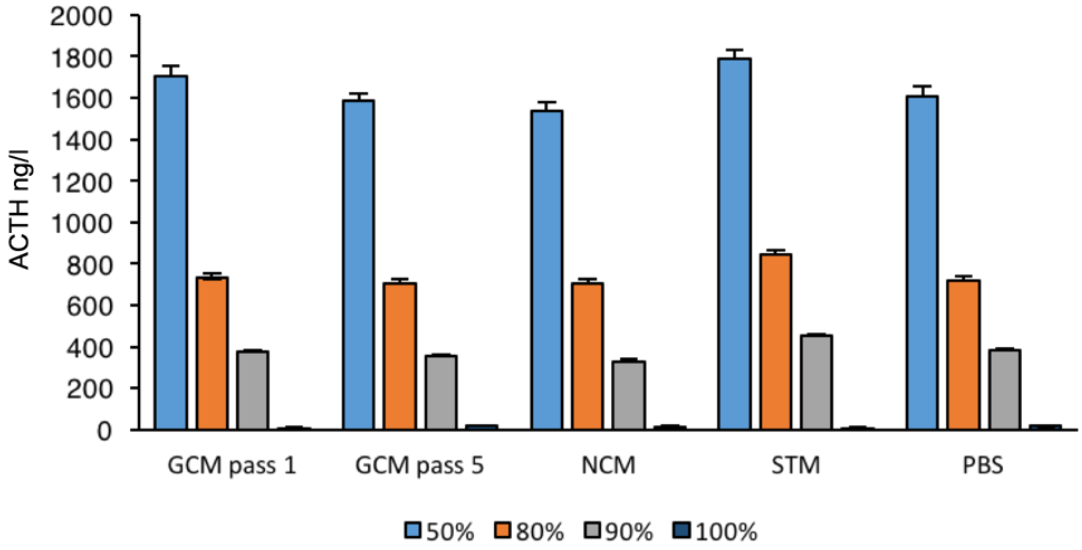

Figure 4.11 Gist culture supernatant does not interfere with the ACTH assay
Serum from a patient with Addison's disease was used as a positive control for the ACTH assay, and diluted with various proportions of Gist culture supernatant (GCM, NCM), standard culture media or PBS. There was no difference in ACTH levels between these samples. GCM pass 1 and 5 refer to supernatant taken from the first and fifth passages of this primary culture. STM – standard GIST culture medium (see methods section 2.1.1 for constituents).

4.4.4 GIST expresses POMC and enzymes required for POMC processing

The possibility that a peptide other than ACTH derived from POMC was responsible for the stimulation of cortisol production *in vivo* and *in vitro* was considered. RT-PCR confirmed expression of POMC, along with the enzymes PC1/3 and PC2 which catalyse the processing of POMC into smaller peptides (Figure 4.12). POMC primers were designed to exon 3 of POMC, which limits the significance of this PCR result however as this would be positive whether transcription was being initiated at the pituitary promoter or the downstream "peripheral" promotor. As described in section 1.6.3, initiation of transcription from the downstream promoter generates an mRNA transcript of roughly

800nt, which lacks the signal sequence for targeting to the endoplasmic reticulum and therefore secretion of the translated peptide. Primers were designed to span intron B (Figure 4.12 C) and were used to demonstrate expression in pituitary tissue and GIST, but this was an inconsistent result and could not be repeated even in the positive control.

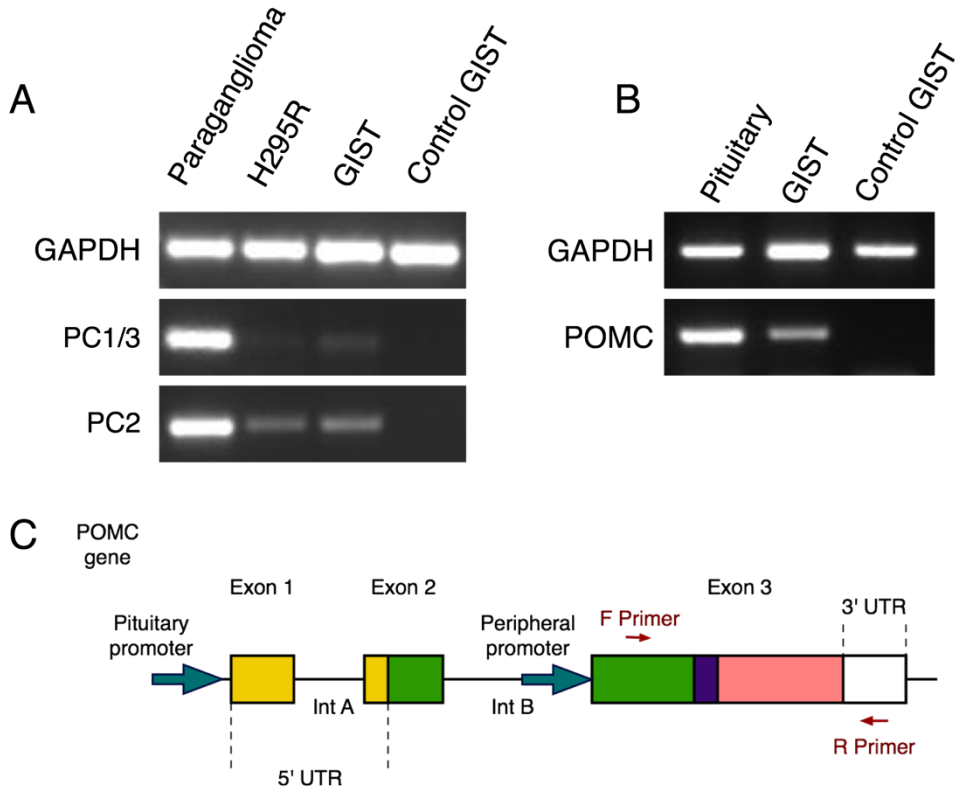

Figure 4.12 GIST expresses POMC and the processing enzymes PC1/3 and PC2
RT-PCR demonstrated POMC expression (A), along with the proteolytic enzymes PC1/3 and PC2 (B) required for processing it to constituent peptides. Panel C shows the primer binding sites on POMC

4.4.5 The presence of alpha-MSH in the GIST tumour is demonstrated by immunohistochemistry

Immunostaining was carried out with antibodies directed against a series of POMC-derived peptides (N-POC 1-29, N-POC 1-49, γ1-MSH, γ-3 MSH, N-terminal ACTH, Mid ACTH, C-terminal ACTH, N-terminal α-MSH, C-terminal α-MSH) (Figure 4.13). Positive control tissues were human pituitary corticotroph adenoma (supplied by the Core Pathology Department, Blizard Institute, QMUL) and an ACTH-secreting lung carcinoid tumour taken from a patient with ACTH-dependent Cushing's syndrome. As a negative

control normal bronchial tissue from the same patient was used. All 9 antibodies exhibited positive immunostaining against the positive controls and negative immunostaining to normal bronchiole (Figures 4.14 and 4.15).

There was no immunostaining of the GC GIST for the N-terminal peptides (Figure 4.16), or for unprocessed ACTH (Figure 4.17). However, almost all cells of the GC GIST immunostained positively using antibodies directed against the acetylated N-terminus and the amidated C-terminus of alpha-MSH (Figure 4.17). There was no immunostaining of the control GIST using any of the POMC antibodies.

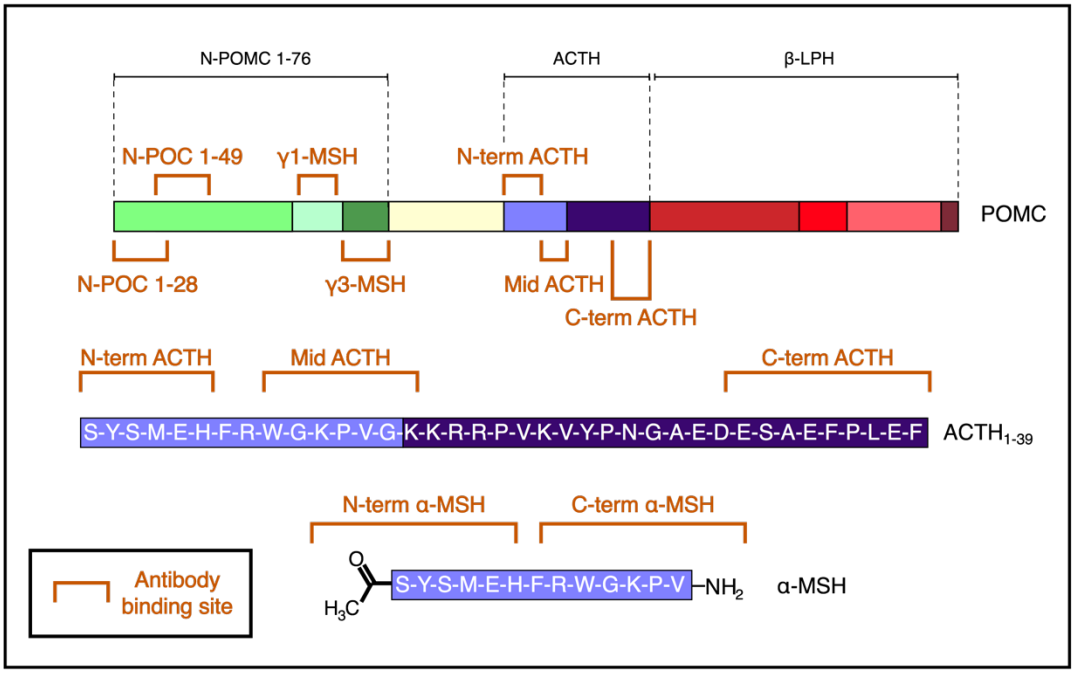

Figure 4.13 Antibody binding sites for POMC peptide immunostaining.
Anti-N-term α-MSH binds only to the acetylated N-terminus of the peptide and not to the N-terminus of ACTH, while Anti-C-term α-MSH binds to the amidated C-terminus, and not to the equivalent amino acid sequence of unprocessed ACTH

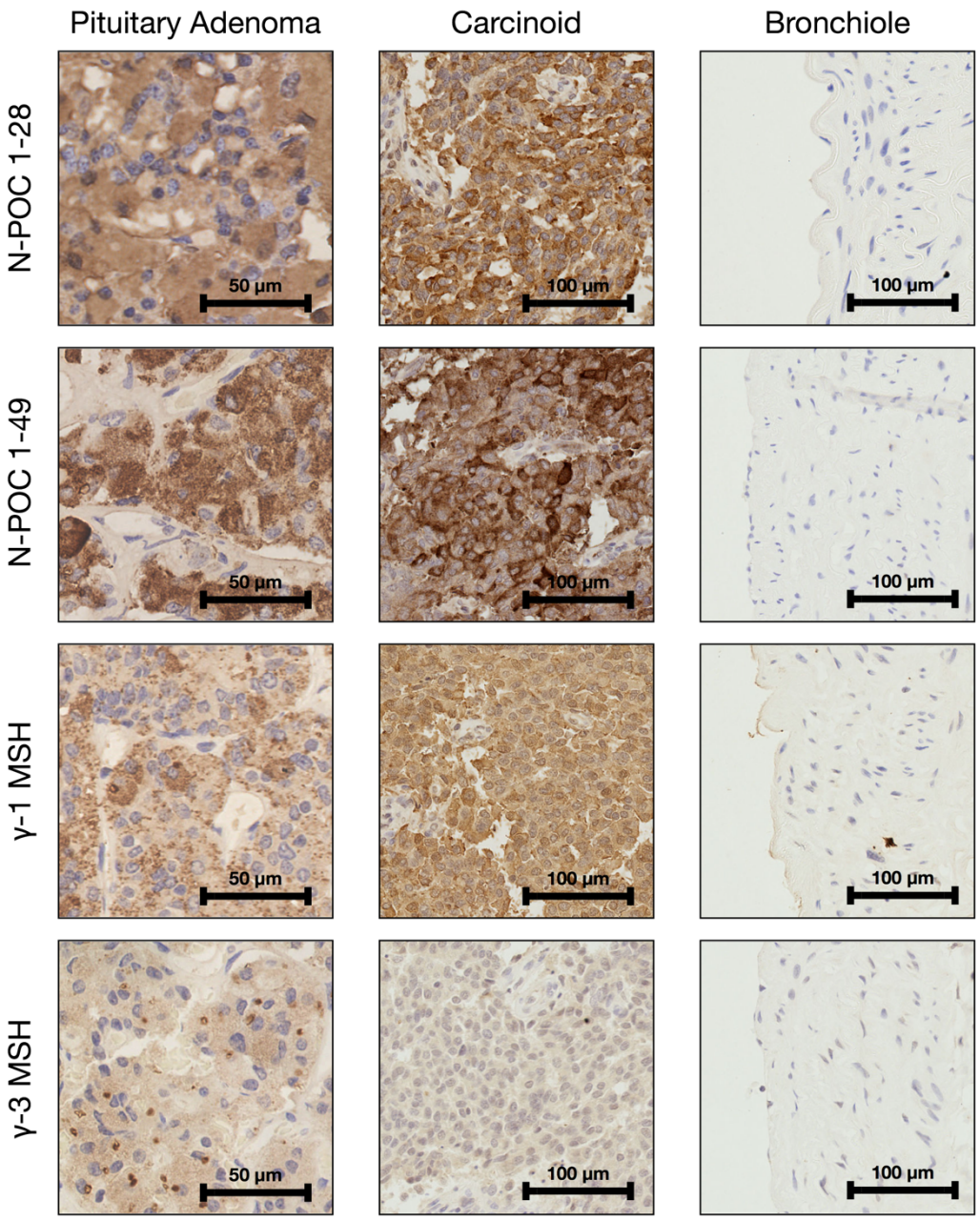

Figure 4.14 N-terminal POMC peptide antibody immunostaining – positive and negative controls. Immunostaining of 3µm sections of human pituitary adenoma and an ACTH-secreting lung carcinoid were carried out according to methods in section 2.6.1. Antibodies were directed at peptides produced from the N-terminal end of POMC (see figure 4.13 for antibody binding sites). Positive immunostaining is demonstrated for all 4 antibodies. A section of bronchiole from normal lung in the patient with the lung carcinoid was used as a negative control and did not show any positive immunostaining.

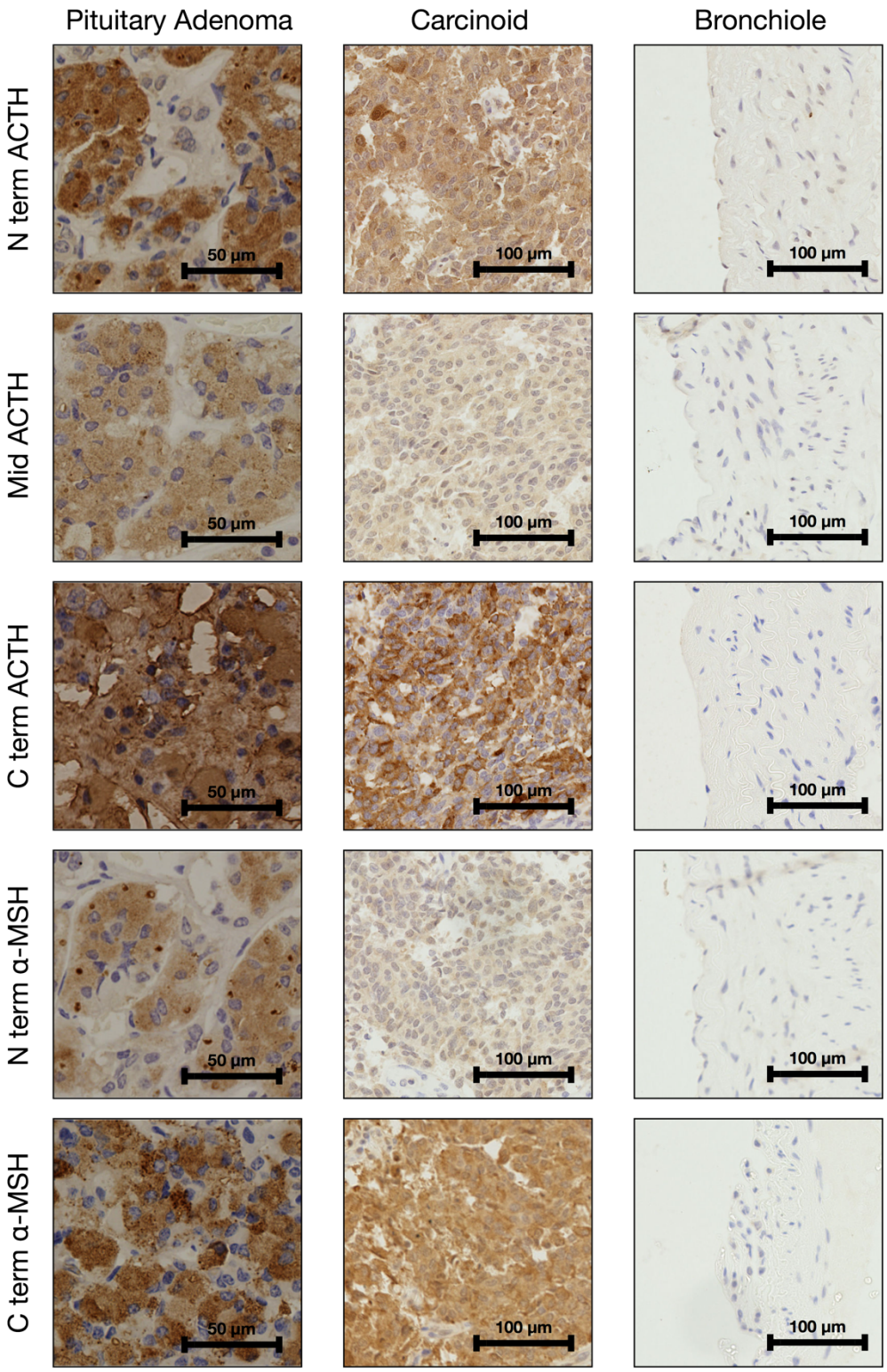

Figure 4.15 ACTH and alpha-MSH immunostaining – positive and negative controls.
Immunostaining of 3µm sections of human pituitary adenoma and an ACTH-secreting lung carcinoid were carried out according to methods in section 2.6.1. Three antibodies directed against unprocessed ACTH and 2 antibodies directed against alpha-MSH (see figure 4.13) all showed positive immunostaining for human pituitary adenoma and ACTH-secreting lung carcinoid. There was no staining of bronchiole from normal lung in the patient with the lung carcinoid.

Overleaf

Figures 4.14 and 4.15

3µm sections of GIST were immunostained according to the method described in section 2.6, table 2.1. Antibody concentrations were as follows: N-POC 1-28 1:2000; N-POC 1-49 1:2000; γ1-MSH 1:2000; γ3-MSH 1:1250; C-term ACTH 1:2000; mid-ACTH 1:1250; N-term ACTH 1:2000; acetylated N-terminus of α-MSH 1:1250; amidated C-terminus α-MSH 1:2000. Positive control for all POMC-peptide immunostaining was a section of human pituitary adenoma (tissue provided by Core Pathology Dept, Blizard Institute, Queen Mary, University of London). Negative control tissue was a GIST from a patient with no evidence of clinical or biochemical Cushing's syndrome. Figure 4.14 demonstrates negative GIST immunostaining for N-terminal POMC peptides. Figure 4.15 demonstrates positive immunostaining for acetylated N-term α-MSH and amidated C-term α-MSH, but negative immunostaining for the three antibodies directed against unprocessed ACTH (N-term ACTH, mid-ACTH, C-term ACTH). Control GIST immunostaining was negative for all POMC peptides.

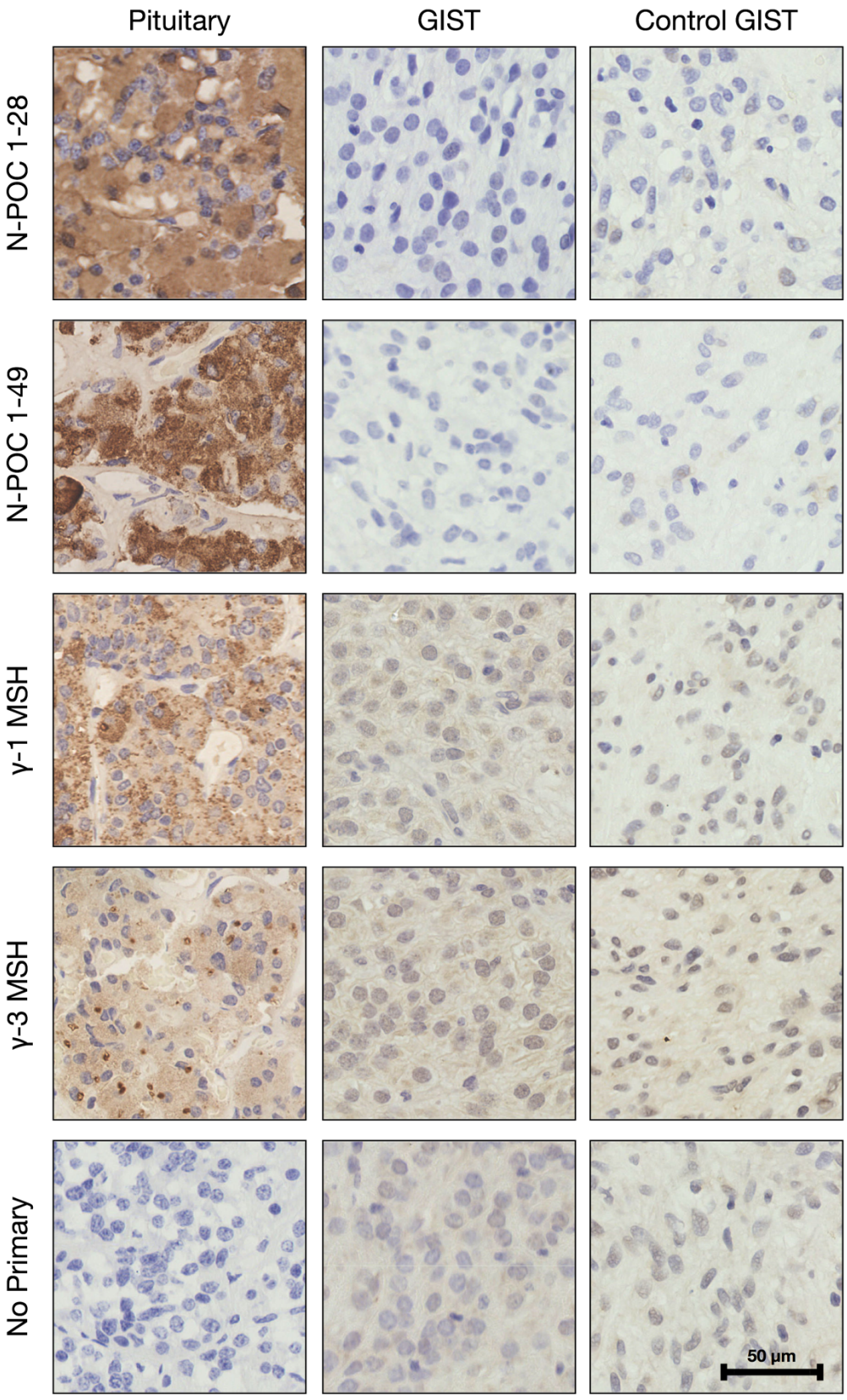

Figure 4.16. GIST immunostains negatively for antibodies directed against N-terminal POMC peptides

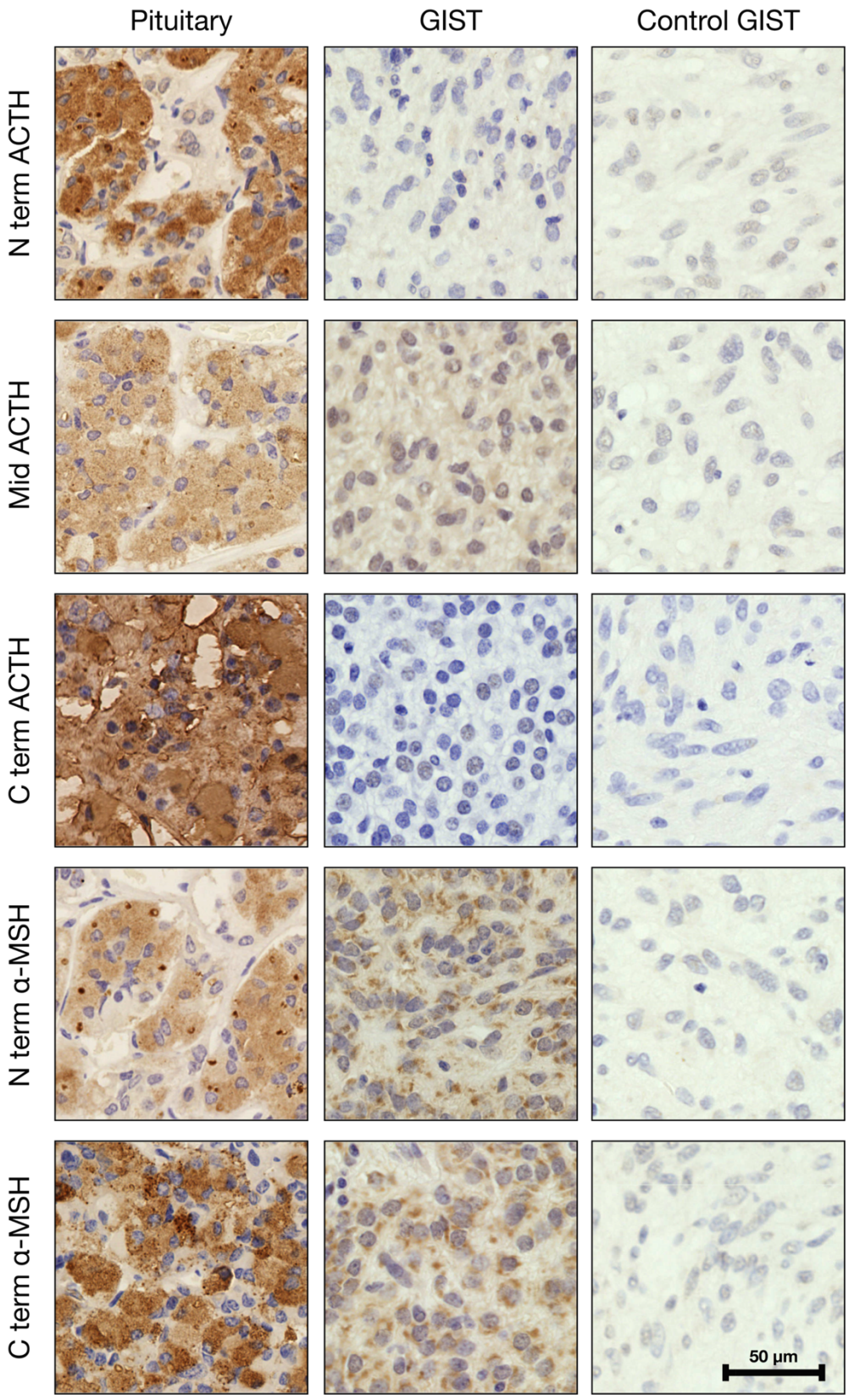

Figure 4.17 GIST immunostains positively for antibodies to α-MSH

4.4.6 POMC expression in GIST is not the result of altered methylation status of the POMC promoter

Bisulphite modification of genomic DNA following by pyrosequencing was performed to assess the degree of CpG methylation of a portion of the pituitary POMC promoter -417 to -260 upstream of the transcription initiation site of exon 1. Three CpG sites reported to be hypomethylated in ACTH-secreting tissues were analysed (Figure 4.15, upper panels) (Ye et al., 2005). Genomic DNA extracted from a corticotroph pituitary adenoma and ACTH-secreting carcinoid tumour demonstrated low levels of methylation at these sites, compared with a non-ACTH secreting carcinoid tumour and both GIST tumours (Figure 4.15, lower panel).

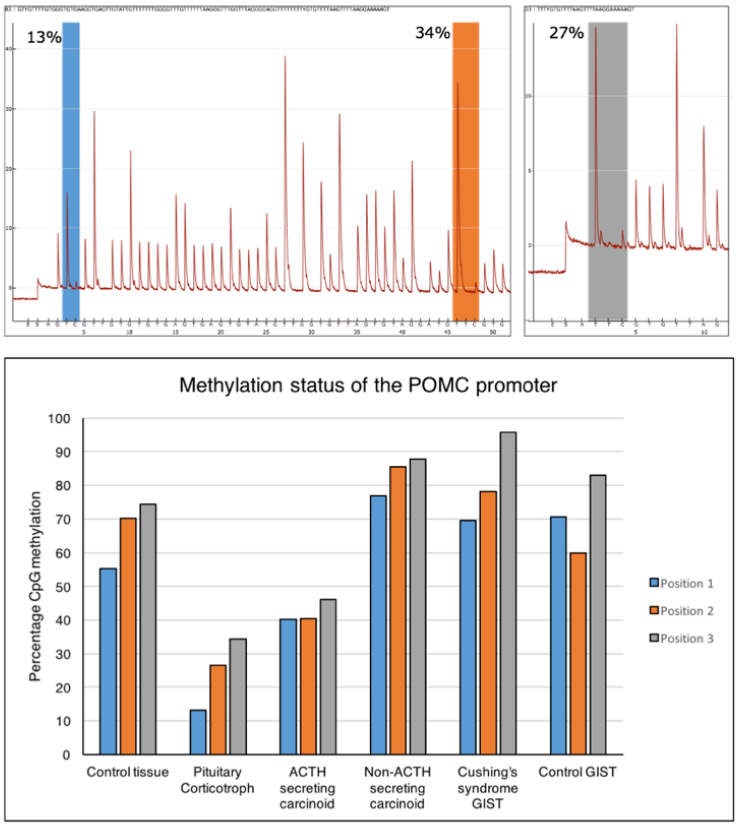

Figure 4.18 The pituitary POMC promoter in GIST tissue is not hypomethylated
The methylation status of the POMC pituitary promotor was assessed by a pyrosequencing method. This region is hypomethylated in pituitary corticotroph cells and in ACTH-secreting neuroendocrine tumours. Genomic DNA was extracted from tissue which was known to secrete ACTH (a pituitary corticotroph adenoma and an ACTH-secreting lung carcinoid tumour), as well as a non-ACTH secreting carcinoid tumour, the Cushing's associated GIST, and a GIST tumour taken from a patient with no clinical or biochemical evidence of Cushing's syndrome. Bisulphite modification and pyrosequencing of a 157bp region which contains three hypomethylated CpG sites in ACTH-secreting tissues (Position 1-3). The percentage of methylation in the GIST tumour was equivalent to that in the non-ACTH secreting tissues.

4.4.7 Alpha-MSH is present in GIST culture supernatant

GIST primary culture supernatant was assayed for alpha-MSH using enzyme-linked immunoassay and two-site sandwich ELISA. Both methods confirmed the presence of alpha-MSH in GIST culture supernatant, but not in standard media or control GIST supernatant (Figure 4.16).

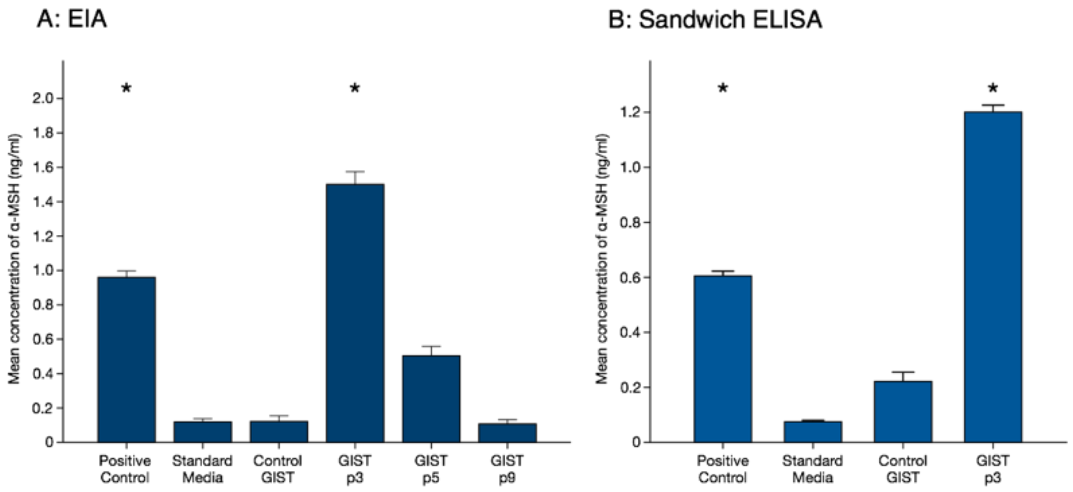

Figure 4.19 GIST culture supernatant contains α-MSH
GIST culture supernatant was assayed for α-MSH using two methods: an enzyme linked immunoassay and a sandwich ELISA (see methods section 2.3.4). α-MSH was detected by both methods. Later passages of GIST (p5, p9) demonstrated lower levels of α-MSH, which would be in keeping with their diminishing in vitro effect on cortisol secretion. ($p<0.05$). "Standard media" is fresh, unused GIST culture media (See methods section 2.1 for full constituents)

4.4.8 Alpha-MSH stimulates the release of cortisol from human adrenal cells in vitro.

The ability of alpha-MSH to stimulate cortisol production from H295R cells and dispersed human adrenal cells was assessed. Initial experiments using alpha-MSH (Sigma) failed to demonstrate any increase in cortisol production. A positive control for the biological activity of alpha-MSH was sought, namely the up-regulation of melanin production in the mouse melanoma cell line B16-F10. A melanin assay was performed as previously described. Alpha-MSH (Sigma) failed to generate an increase in melanin production, whereas exposure to ACTH 1-39 (Sigma) did lead to elevated melanin production (Figure 4.17).

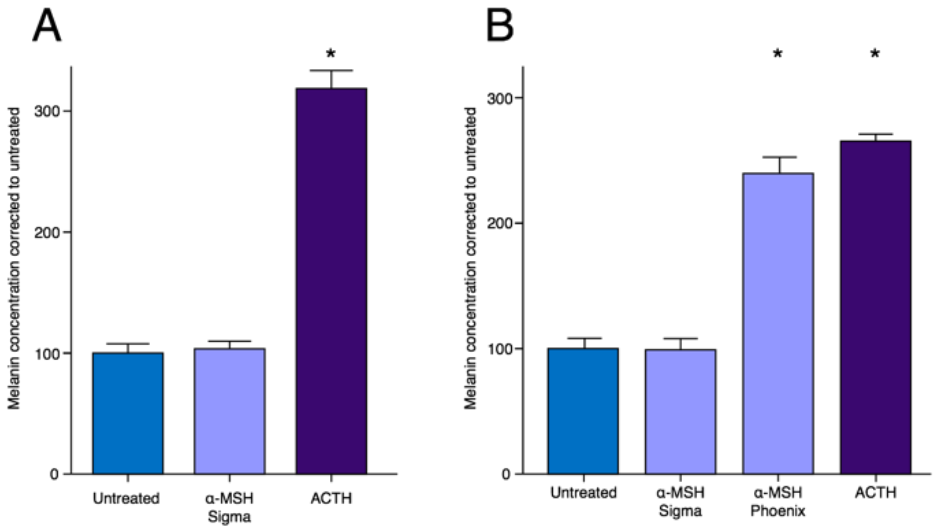

Figure 4.20. Alpha-MSH from one supplier failed to stimulate melanin production from mouse melanoma cells
B16F10 mouse melanoma cells were stimulated overnight 1μM ACTH 1-39 or 1μM α-MSH in a DMEM plus 10% FBS. Cells were trypsinised, resuspended and pelleted, before resuspension in 1ml 1N NaOH. Absorbance was measured at 420nm and melanin concentration was determined with reference to a standard curve of synthetic melanin in the range 1-100μg/l, and corrected for the protein content of the cell lysate. Panel A shows that α-MSH purchased from Sigma did not stimulate melanin production, but α-MSH purchased from Phoenic Pharmaceuticals did result in melanin production equivalent to ACTH stimulation. Experiments in triplicate, $p<0.05$.

A second supplier of synthetic alpha-MSH was sourced - Phoenix Pharmaceuticals. Contrary to the Sigma-supplied peptide, the specification sheet for the Phoenix peptide instructed that it should be used immediately on first making up a working solution, and not frozen. On following these instructions, the biological activity of the peptide was assessed using the melanin assay, and an increase in melanin production was demonstrated (Figure 4.17 B). Applying this second alpha-MSH peptide to dispersed human adrenal cells and H295R cells confirmed the ability of alpha-MSH to stimulate cortisol release from human cells in vitro, independent from ACTH (Figure 4.18).

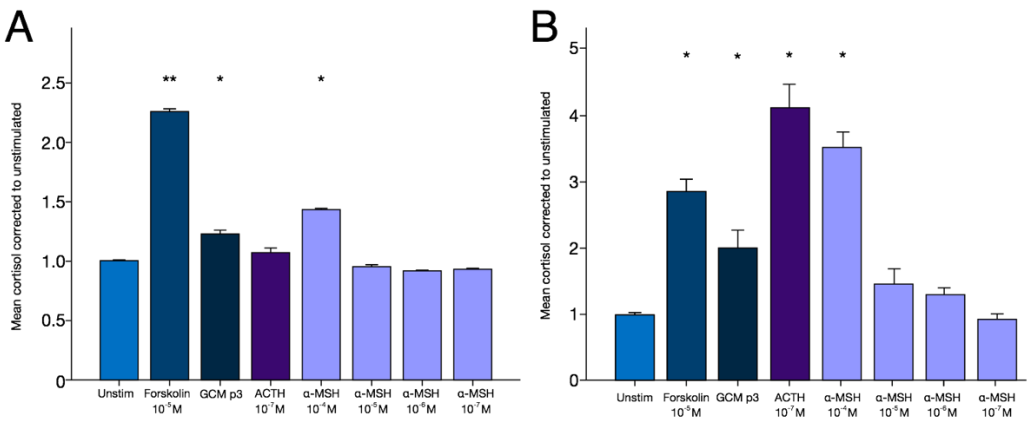

Figure 4.21 Alpha-MSH can stimulate cortisol production from H295R and human adrenal cells in primary culture
H295R cells (A) and human adrenal cells in primary culture (B) were stimulated overnight with incubated with various concentrations of alpha-MSH and ACTH 1-39, and GIST culture supernatant from the third passage of the primary culture (GCM p3). Forskolin was used as a positive control for cAMP-mediated signalling. H295R cells (A) demonstrated a significant increase in cortisol production in response to forskolin, GCM, ACTH and α-MSH 10-4M. Primary culture of human adrenal cells also exhibited statistically significant increases in cortisol production when incubated with these Forskolin, ACTH, GCM and α-MSH 10-4M. There was a trend towards increasing cortisol production with increasing concentrations of α-MSH which did not become statistically significant until the highest 10-4M dose was reached.

4.5 Discussion

4.5.1 Clinical Assessment

The patient described was clinically very severely Cushingoid, with hypertension, osteopenia and weight gain in a typical centripetal distribution, and her clinical photographs show a typical appearance. The osteopenia may have contributed to her low impact metatarsal fracture. The pigmented appearance had not gone unnoticed by the patient, who reported that friends had remarked on her tanned appearance, and that this was unusual for her as she had never previously had a suntan, rather she developed freckles.

The a priori likelihood that any patient with Cushing's syndrome has ACTH-dependent disease is high, with the majority of cases the result of a corticotroph adenoma of the pituitary. Skin pigmentation in the context of Cushing's syndrome increases the clinical suspicion for the ectopic ACTH syndrome, where serum levels of ACTH can reach much higher levels (Alexandraki & Grossman, 2010). The possibility that ectopic ACTH

secreting neuroendocrine tumours are secreting a wider variety of peptides with the ability to upregulate the tanning process may also contribute to this (Lowry, 2016).

It was unexpected therefore that biochemical assessment demonstrated low levels of ACTH in the serum. Assessment of her cortisol axis confirmed the loss of circadian variation in cortisol secretion, with failure to suppress with LDDST. It is important to note that initial assessment of cortisol axis was performed while the patient continued to apply her transdermal oestrogen patch. Oral oestrogen therapy must be stopped 8 weeks in advance of cortisol assessment due to its effect of increasing the levels of cortisol binding globulin (CBG) - transdermal oestrogen is not subject to first pass metabolism in the liver and therefore does not have the same effect on CBG

Radiological investigation demonstrated bilateral macronodular adrenal hyperplasia. A comprehensive biochemical assessment for ACTH-independent macronodular adrenal hyperplasia (AIMAH) has been recommended by Lacroix *et al* (Lacroix et al., 1999) on the basis that expression of a diverse range of non-mutated membrane bound receptors within the adrenal cortex is implicated in the regulation of steroidogenesis. Gastric inhibitory polypeptide (GIP) expression is one of the best recognised causes of AIMAH, and can be assessed by the response of cortisol to a mixed meal test. Treatment with somatostatin analogues has been used to treat this food-dependent Cushings. For these reasons both a mixed meal test and an octreotide test dose were given, but without any change in cortisol secretion. LH-receptor expression can be assessed by the response to GnRH administration, but our patient's LH and FSH had been suppressed by exogenous oestrogen, so this test was not performed. Moving from a supine to upright posture can stimulate cortisol secretion when it is being controlled by expression of vasopressin receptors, the β-adrenergic receptor or the angiotensin receptor. Expression of the 5-HT4 receptor causing Cushing's can be assessed by the response to the 5HT-4 agonist metoclopramide. These tests were not performed in this case. A CRH test was performed, but there were no changes in ACTH or cortisol in response to its administration.

A failure to fully investigate for all possible mechanisms of AIMAH can be at least partly explained by the radiological appearance of the adrenal glands in this case. The large size of the right adrenal gland, and the slight heterogeneity noted on CT raised the possibility of a low grade adrenocortical cancer which would require adrenalectomy. It is not

uncommon for the adrenal enlargement in AIMAH to be asymmetrical, as in this case (Swain et al., 1998). This can sometimes lead to an erroneous diagnosis of unilateral adrenal pathology. On the other hand, unilateral adrenalectomy has been recommended in selected patients as a treatment for both AIMAH and PPNAD (Xu, Y. et al., 2013).

The possibility that the GIST could be secreting some agent which was stimulating the adrenal gland was considered. There are isolated reports of GISTs secreting IGF-II and leading to clinical hypoglycemia, and in vitro studies demonstrating expression of receptors for somatostatin, bombesin, cholecystokinin and vasoactive intestinal peptide (Reubi et al., 2004). In addition there is evidence of a neuroendocrine phenotype in the expression of synaptic vesicle proteins including synaptobrevin, synapsin 1 and amphiphysin (Bumming et al., 2007), while a large proportion of GISTs have been shown to express ghrelin (Ekeblad et al., 2006). There have been no reports in the literature of GISTs causing Cushing's syndrome, or any other endocrine disease. However, the absence of an obvious genetic syndrome to link GIST and AIMAH either in the literature, or from clinical examination, suggested a possible link at some level, and it was hypothesised that this could be related to hormone secretion from the GIST. From a theoretical perspective, removal of the GIST alone would have been correct operation to test this hypothesis, but as described, concerns about the size of the right adrenal led to a compromised decision.

As described, GIST tumours are characterised by mutations in the KIT oncogene (75-80%) or in the platelet derived growth factor A (PDGFRA; app 10%)(Lasota & Miettinen, 2008). Mutations of KIT and PDGFRA promote oncogenic signalling through the mitogen-activated protein kinase (MAPK) and phosphoinositide (PI3K) pathways (Joensuu et al., 2013). CD117 is an alternative name for KIT, and used as an immunohistochemical marker for GISTs. Staining for CD117 and DOG-1 confirmed that the gastric tumour was a GIST. DOG-1 is a chloride channel with a sensitivity of 94.7% in the identification of GISTs, and is now widely used alongside CD117 (Miettinen et al., 2009).

The histopathology of the excised adrenal gland will be considered in detail in Chapter 5. It is worth noting here that a diagnosis of PPNAD was raised, although the patient was not in the typical age range for new onset of PPNAD, and the adrenals are usually small with micro nodules in this condition(Travis et al., 1989). However, this possibility was considered as discussed later,

She was treated for a short period of approximately 4 weeks with metyrapone, with a marginal improvement in her cortisol burden, but immediately following the surgery to remove the GIST and the right adrenal gland, her cortisol levels dropped significantly. Her 9am cortisol 3 days' post-surgery was not undetectable however, but this is likely to reflect replacement hydrocortisone from the previous day, rather than endogenous cortisol production. On the basis of this result, the possible mechanisms for her Cushing's are as follows.

- Secretion of some agent from the GIST which is stimulating cortisol secretion from the adrenal glands. Elevated cortisol secretion has suppressed pituitary ACTH secretion, so after removal of the GIST there is insufficient ACTH to stimulate cortisol secretion from the remaining left adrenal gland.

- Cortisol secretion was due entirely to autonomous secretion from the larger right adrenal. Elevated serum cortisol would suppress pituitary ACTH, and therefore on removal of the right adrenal, there would again be insufficient ACTH to stimulate cortisol production from the left adrenal. One might expect the left adrenal to be atrophied rather than hyper plastic and nodular in this situation, however. Furthermore, the shrinkage in the size of the left adrenal in the three years following removal of the GIST suggests that it has lost some stimulus to its growth.

- AIMAH with asymmetrical enlargement of the right adrenal gland, due to ectopic expression of one of the receptors described above. It may be expected that the left gland would continue to secrete cortisol following the removal of the right this instance, as the stimulus for cortisol production would still be present. However, chronic stimulus to the right adrenal could lead to it developing autonomous secretion as described above.

The resolution of her pigmentation following surgery is difficult to explain on the basis of removal of the adrenal gland alone, without invoking some additional stimulus to pigmentation. However, it is worth noting that in cases of ACTH driven hyperpigmentation such as Nelson's syndrome or Addison's disease, the skin pigmentation is more widespread than seen in this patient, where it was apparent in the sun-exposed

areas. One might explain this by invoking the stimulus for skin pigmentation as being the sum total of melanocortin 1 receptor activation by ACTH or alpha-MSH *plus* the degree of UV exposure. UV light stimulates local MSH production, but also numerous keratinocyte derived factors such as NGF, endothelin-1 and bFGF, which are involved in the tanning response and synergise with the results of MC1R activation (Gilchrest et al., 1996). This will be described in more detail in Chapter 5. As described, she had an MC1R polymorphism that is associated with reduced activity on ligand binding, but not zero activity. The proposed high serum levels of alpha-MSH, along with direct UV exposure can therefore be hypothesised to result in hyperpigmentation only in sun-exposed areas.

This patient developed a seronegative arthritis following the resolution of her Cushing's syndrome. The development of overt immune dysfunction is well described in the literature following treatment for both ACTH-dependent and ACTH-independent disease, with reports of patients developing conditions including sarcoidosis, psoriasis, and systemic lupus erythematosus (da Mota et al., 2011). This is generally defined as a rebound phenomenon in the absence of previously high cortisol levels. However, the direct anti-inflammatory actions of melanocortins is now a well-recognised phenomenon (Giuliani et al., 2010), and the consequences of removing a long term stimulus from ACTH, or perhaps alpha-MSH, may also be involved, and an interesting area for future work. The suppression of ACTH post-operatively as a result of prednisolone treatment makes it difficult to attribute the reduction in size of the left adrenal entirely to the removal of a putative adrenal growth stimulus from the GIST.

4.5.2 Negative controls

A comprehensive examination of the endocrine capabilities of GISTs would require much larger numbers of samples. The lack of previous reports of GISTs causing Cushing's syndrome, and the relative rarity of these tumours locally led to the decision to use a GIST from one patient with no evidence of Cushing's clinically or biochemically. A more suitable negative control GIST would have been one which shared an identical histological and immunohistochemical profile with the GIST from the patient with Cushing's syndrome.

4.5.3 Sequencing and genetic syndromes

The presence of a previously reported PDGFRA mutation (D842V) in tumour but not leucocyte DNA identifies this as a sporadic GIST, in common with most of these tumours. This mutation is by far the most common PDGFRA mutation, and is associated with a slight reduction in CD117 immunohistochemistry, and a more epithelioid morphology in the tumour, as was demonstrated in this case(Lasota et al., 2004).

We also assessed for loss of function in the succinate dehydrogenase (SDH) complex, which is present in approximately 75% of gastric GISTs, especially in children and young adults (Janeway et al., 2011). Loss of any of the subunits is immunohistochemically observed through loss of SDHB expression, but mutations in all of the subunits has been described(Dubova et al., 2015). There was normal SDHB expression in this case. The familial syndromes of Carney's triad (GIST, paraganglioma and pulmonary chondroma) and Carney-Stratakis syndrome (GIST and paraganglioma) are among the SDH-deficient GISTs. Neurofibromatosis type 1 is associated with GISTs but there was no clinical suspicion of this syndrome in this case (Andersson et al., 2005). A small set subset of GISTs harbour a BRAF mutation (V600E), but was not present in this case (Miettinen & Lasota, 2013).

Sequencing was also carried out to look for common mutations associated with PPNAD. This condition is characterised by small to normal sized adrenals containing multiple black/brown nodules within atrophic surrounding cortex (Horvath, A. & Stratakis, 2007). It may occur independently, but 90% of cases are part of the Carney complex (CNC). This multiple tumour syndrome is associated with a variety of pigmented lesions, including a characteristic spotty skin pigmentation, melanotic schwannomas, and epithelioid blue nevus, in addition to cardiac myxomas and adrenocortical and pituitary adenomas (Carney et al., 1985).

CNC and PPNAD are characterised by up-regulation of intracellular cAMP mediated protein kinase A signalling pathways. As previously discussed, this is the pathway that mediates the effects of melanocortin receptor binding. It is therefore apparent that excess cortisol production from the adrenal gland may be a consequence; a link to the possible mechanism for pigmentation will be discussed in Chapter 5. Mutations in the PKA regulatory subunit type 1 alpha (PRKRA1A) lead to a functional excess of PKA

signalling(Kirschner et al., 2000). Phosphodiesterase enzymes including PDE11A and PDE8B catalyse the hydrolysis of cAMP and cGTP and down-regulate the PKA pathway, and mutations in both of these have been demonstrated in CNC and PPNAD (Horvath, A. et al., 2006).

PRKAR1A and PDE11A were sequenced in the adrenal and leucocyte DNA in this case and found to contain no mutations. PDE8B was not sequenced, but on the basis of clinical and histopathological behaviour a decision was taken that further sequencing was not indicated.

A recent study identified mutations in the tumour suppressor gene ARMC5 in a substantial proportion of patients with AIMAH, with most having both a germline and somatic mutation (Assie et al., 2013). The function of ARMC5 is unknown at this point, but cases with mutations included those with ectopic expression of receptors as described previously. Sanger sequencing for mutations in ARMC5 detected no mutations in adrenal or leucocyte DNA.

4.5.4 Primary culture experiments

Primary cultures of both GISTs were established. To ensure that the cells in culture were GIST rather than fibroblasts they were successfully stained for CD117 and DOG-1. The supernatant from these cultures was used to incubate NCI-H295R cells. The NCI-H295 cell line was established from an adrenocortical cancer, but is an imperfect i*n vitro* model for adrenocortical steroidogenesis in that although it expresses the enzymes necessary for steroidogenesis, it expresses the MC2R only weakly, and therefore does not respond significantly to ACTH (Rainey et al., 2004). It is widely available however, and was used in the preliminary cell culture experiments.

Cortisol production in H295R cells remains driven by the PKA signalling pathway, and therefore the adenylyl cyclase activator forskolin is used as a positive control. Incubation with GIST culture supernatant resulted in a significant increase in cortisol production from H295R cells, greater than that induced by forskolin, although with supernatant from later passages this effect diminished. H89 abolished the effect of GIST supernatant, suggesting that its effect is mediated by the PKA signalling pathway. Pre-heating the media abolished this effect, suggesting that the active ingredient was a peptide.

Upregulation of SF-1 and StAR by GIST supernatant was demonstrated by quantitative PCR, in keeping with the increase in cortisol secretion. A lack of CYP17A1 expression in rats and mice means that DOC is the substrate for both p450c11β as well as p450c11AS and therefore the main glucocorticoid produced in these species is corticosterone (Miller & Auchus, 2011). An increase in corticosterone secretion from these cells was further evidence that GIST supernatant contained some agent, most likely a peptide, with the ability to stimulate cortisol production *in vitro*. The hypothesis that the agent responsible for cortisol secretion is alpha-MSH could be further tested by including one or both of the anti-alpha MSH antibodies in the cell culture medium when GIST culture supernatant is applied to H295R, rat adrenal cells or human adrenal cells. This would be expected to bind the alpha-MSH and result in a diminished cortisol response. As discussed however, the cortisol response diminished with each GIST culture passage, so there was insufficient active media to perform this experiment effectively. Additional negative control GIST cultures and cultures from other non-GIST tumours would also be valuable to ensure that the active agent was not some non-specific feature of conditioned media. For instance, the early passages of any cell culture might be expected to include more fat-containing cells from the original tumour. Cholesterol released directly from the cultured cells into the media could therefore serve as a substrate for cortisol production within the adrenal cells. Other agents that might stimulate cortisol production include cAMP released directly into culture supernatant. There are a number of methods for assaying cAMP, including ELISA, and these could be performed on aliquots of various culture supernatants prior to their use in stimulating H295R cells.

4.5.5 Assay interference

Notwithstanding the absence of ACTH in the patient's serum and in GIST conditioned media, the possibility of ACTH being the stimulus for cortisol production in both patient and tissue culture remained. The possibility that the GIST was producing ACTH, but that for some reason this was not being detected by the two site ACTH electrochemiluminescence immunoassay, was considered. This can occur with ectopic ACTH secreting tumours in a number of circumstances. If they are secreting ACTH which is substantially phosphorylated at the Ser31 residue this can interfere with the binding of the C-terminal antibody, and underestimate the total amount of ACTH present. This form of ACTH is biologically active, but whereas only 20-30% is phosphorylated in the pituitary, the bulk may be phosphorylated in ectopic tumours if they are processing POMC

more like the intermediate lobe of the pituitary (Massias et al., 1994). Such tumours may also process ACTH further, producing large amounts of CLIP. This, too, can bind the C-terminal antibody in the sandwich assay and lead to inaccurate readings of the intact ACTH 1-39 peptide (Lowry, 2016).

A positive control ACTH sample taken from a patient with Addison's disease prior to their morning dose of hydrocortisone was used, and various proportions of GIST culture supernatant was added, but this failed to alter the assay result compared with PBS or standard culture media. An additional means to assess for ACTH in the patient's serum would have been to send it to another laboratory to be run on a different platform. Unfortunately, stored serum samples were inadvertently disposed of.

4.5.6 Strategies for identifying active component of GIST culture supernatant

At this point a number of strategies were considered to enable identification of the component of the culture supernatant responsible for stimulation of cortisol secretion. The likelihood that this was a peptide led to an initial plan to fractionate the conditioned media using a combination of standard protein purification techniques, including sequential ion-exchange, reverse phase and size exclusion chromatography. The various fractions would be assayed for their ability to stimulate cortisol production from H295R cells *in vitro*. Active fractions would be pooled and further purified, before SDS-Page gel electrophoresis to separate out peptide components. The aim would be to have fewer than 10 bands on Coomassie staining. The bands would be excised and subjected to mass spectrometric analysis to identify them. Those proteins which were present in the media from the GC supernatant but not the negative control NC supernatant would be purchased, and applied to H295R cells to test their activity. Any potential factors which were not commercially available would be generated by expression of cloned DNA in a bacterial vector.

For a number of reasons, it was decided not to pursue this strategy. As noted previously, the supernatant from the primary culture was stimulating cortisol to a lesser extent with successive passages. A change in phenotype of primary cells in culture is well recognised, so this is not surprising (Wolffe & Tata, 1984). The volumes of most active supernatant from the early passages were limited, and would likely have been insufficient for the

experiments described. Furthermore, the expertise to carry out the protein purification was not immediately available within the laboratory. Therefore, an alternative hypothesis was developed.

4.5.7 POMC

The steroidogenic effects of POMC-derived peptides other than ACTH 1-39 was briefly discussed previously. The γ-MSH peptides have been demonstrated to potentiate the adrenal response to ACTH by increasing the activity of hormone sensitive lipase (HSL). This breaks down cholesterol esters to free cholesterol that can be used in the steroidogenic pathway when ACTH has bound the MC2R. The N-terminal fragment N-POMC is co-secreted with ACTH from the anterior pituitary, and it has been shown that antibodies directed at it can block compensatory growth following adrenalectomy in the rat. The smaller peptides N-POC 1-28 and N-POC 2-54 have also been shown to be potent mitogens to adrenocortical cells *in vitro* (Lowry et al., 1983). The latter are generated from the action of a serine protease that is expressed in the outer adrenal cortex of the rat (Bicknell et al., 2001). Additionally, proliferation of H295R cells has been induced by synthetic N-POC 1-28, via an activation of the ERK signalling pathway (Fassnacht et al., 2003) We therefore sought to establish the presence of POMC-derived peptides in the GIST.

POMC processing is demonstrated in figure 4.19B. Proprotein convertases are widely expressed in endocrine tissues and catalyse the cleavage of prohormones including proPTH and proinsulin. The serine proteases PC1/3 and PC2 are the family members involved in the processing of POMC. PC1/3 is the predominant enzyme in the anterior lobe, while in the intermediate lobe both PC1/3 and PC2 are expressed (Zhou et al., 1993). This is the molecular basis for the generation of predominantly ACTH from anterior pituitary, while smaller POMC peptides such as α-MSH are produced in the intermediate lobe. Expression of both proprotein convertases in the GIST tumour is necessary for complete POMC processing to alpha-MSH, and it is interesting to note that the PCR band for PC2 is more intense than the PC1 band, suggesting that there is higher PC2 expression within the GIST. This would be expected if we are proposing that it is behaving more like a melanotroph cell than a corticotroph cell, but a quantitative RT-PCR approach would be necessary to make any definitive statements on this point. Expression

of POMC was demonstrated using RT-PCR, although this alone does not establish that the GIST is capable of secreting a processed alpha-MSH peptide, as will be outlined below.

The nine antibodies used to stain for unprocessed and processed POMC were obtained from Dr Andrew Bicknell (University of Reading) but had not been used for immunohistochemistry previously. Positive control tissues used were human corticotroph pituitary adenoma, and a lung carcinoid tumour from a patient with ACTH-dependent Cushing's syndrome. Although it is recognised that ectopic-ACTH secreting tumours may process and secrete alpha-MSH peptides (Roth et al., 2008), the validity of using human pituitary adenoma tissue as a positive control is not immediately apparent. The lack of a distinct intermediate lobe in the human pituitary (Horvath, E. et al., 1999), and an absence of circulating alpha-MSH in normal circumstances points to an absence of PC2 expression in the anterior pituitary (Iino, K. et al., 2010), although there are some reports of PC2 expression in the normal human pituitary (Hook et al., 2009; Lloyd et al., 1995).

The presence of PC2 expression and consequent alpha-MSH secretion in pathological pituitary conditions is more widely accepted, with PC2 expression reported in pituitary adenomas (Jin et al., 1999; Iino, K. et al., 2010), and the presence of smaller POMC-derived peptides (including alpha-MSH) detected in the serum of patients with pituitary dependent Cushing's disease (Colao et al., 1993; Coates et al., 1986). Increased levels of plasma alpha-MSH have been reported in patients with Addison's disease, hinting at the capacity for pituitary production of this peptide (Donahoo et al., 2009), while infusion of corticotrophin-releasing hormone has been shown to increase alpha-MSH levels in the inferior petrosal sinuses in a subset of patients with Cushing's disease (Colao et al., 1996). It was felt, therefore, that pituitary *adenoma* was a reasonable choice as a positive control for alpha-MSH. However, the use of malignant melanoma tissue as an additional positive control would be valuable.

Immunohistochemical analysis of the GIST tumour using antibodies directed against specific short peptide sequences within POMC demonstrated the expression of alpha-MSH, with positive staining for its acetylated N-terminus and amidated C-terminus, but negative staining for ACTH. These antibodies specifically targeted the post-translationally modified peptides, as demonstrated in figure 4.19C, explaining the lack of staining for N-

terminal ACTH and mid-ACTH. The mid-ACTH antibody targets the same amino acid sequence as C-terminal alpha-MSH without the amide group. The absence of C-terminal

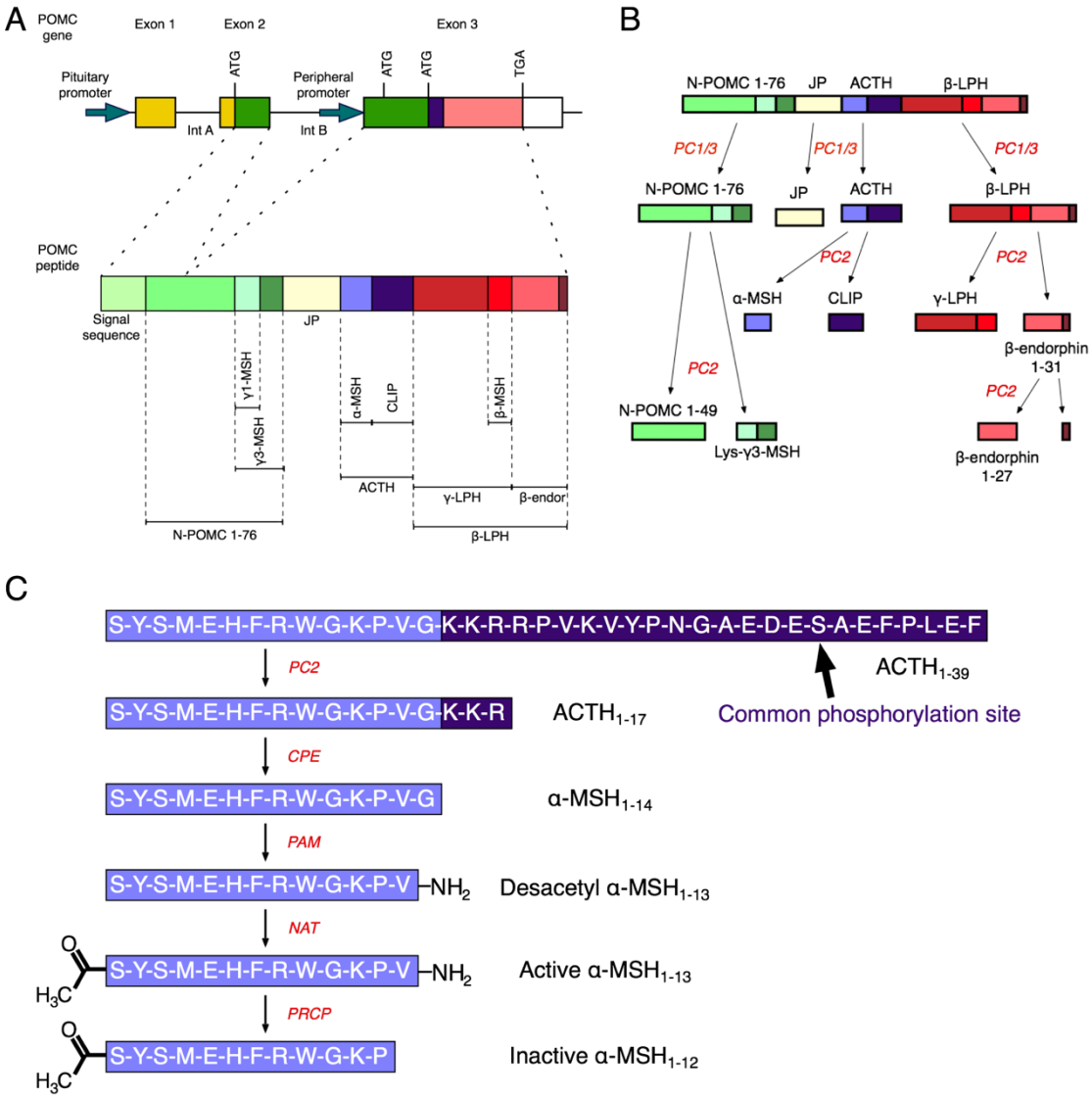

Figure 4.22 POMC processing

A: The POMC gene consists of three exons and two introns. A pituitary selective promoter produces a transcript of 1200nt, in extrapituitary tissues a downstream promoter can produce 800nt transcripts which can potentially be translated from an ATG site just upstream of ACTH.

B: The POMC proprotein convertase processing enzymes PC1/3 and PC2 are differentially expressed in the anterior and intermediate lobes of the pituitary. PC1/3 expression alone will generate ACTH, but high levels of PC2 in the intermediate lobe lead to the production of alpha-MSH

C: The production of alpha-MSH proceeds through a number of additional steps, producing an active 13 amino-acid peptide which is post-translationally modified by the addition of an acetyl group at the N-terminus and an amide group at the C-terminus. Desacetyl-α-MSH is thought to be more active within the adrenal, but less active in the skin than alpha-MSH. CPE –carboxypeptidase E; PAM peptidyl glycine alpha-amidating mono-oxygenase; NAT n-acetyl transferase; PRCP. Phosphorylation at Ser31 on ACTH does not interfere with its biological activity but does interfere with antibody binding to the C-terminus, interfering with measurement in assays and immunohistochemistry

ACTH staining is more difficult to explain, as the CLIP peptide should be present as a result of ACTH processing to alpha-MSH. This could relate to phosphorylation at the Ser31 residue mentioned previously, which interferes with the C-terminal antibody binding in the ACTH sandwich assay. Immunohistochemical staining for the phosphorylated peptide would be informative here.

The lack of N-POMC staining using antibodies directed at the N-terminal POMC peptides (N-POC 1-49, N-POC 1-28, γ3-MSH and γ1-MSH) is surprising given the positive staining for alpha-MSH. Further post-translational modification of these peptides such that the antibody binding sites are obscured or destroyed, or degradation of the peptides entirely within the cells of the GIST could be one mechanism for this. A further, and perhaps more plausible mechanism relates to alternative splicing of POMC RNA.

The POMC gene is 8-kb and located on chromosome 2p23 in the human, and consists of three exons and two intervening introns. The first exon encodes a leader sequence, the second encodes the signal initiation sequence and the N-terminal portion of POMC, and the third encodes most of the mature peptide sequences. A pituitary selective promoter generates an mRNA transcript of approximately 1200nt. In extra pituitary tissues a downstream pituitary promoter is capable of generating truncated 800nt transcripts arising from the 5' end of exon 3 (Giraldi et al., 2011). An additional upstream promoter (not shown in the figure) can generate longer transcripts of approximately 1350nt, particularly in the ectopic ACTH syndrome (Clark et al., 1990)

Clark *et al* demonstrated the expression of a truncated POMC mRNA lacking the first two exons by Northern blot analysis of ACTH-secreting pancreatic NETs (Clark et al., 1989). Demonstration of three different mRNA transcripts of POMC has been demonstrated in human placental tissues, a pituitary like 1200 base transcript, as well as 800 and 1500b species (Grigorakis et al., 2000). Smaller POMC-like mRNA transcripts have also been shown in the testes and ovaries of rat, mouse and monkey (Chen, C. L. et al., 1986). Both truncated POMC mRNA transcripts and immunohistochemical ACTH staining have been demonstrated in human leukaemia cell lines (Murao et al., 1998). Translation of these shorter peptides can potentially be initiated from the methionines (ATG) indicated in

figure 4.19A, as demonstrated in human dermal fibroblasts, but they would lack the signal sequence for targeting to the endoplasmic reticulum, and it is therefore unlikely that they would be processed and secreted (Jeannotte et al., 1987). The primers used in this case were designed to exon 3 of POMC. For the reasons outlined above, it does not follow from the positive PCR result that a POMC peptide would be processed and secreted from GIST tissue on this basis alone. As noted, an intron-spanning approach to primer design would have been preferable, but appropriate primers could not be optimised reliably even on positive control pituitary cDNA.

It will be necessary to sequence the POMC gene in full using a 5' RACE approach to answer these questions(Yeku & Frohman, 2011). This method would allow us to obtain full length cDNA of the entire transcript using primers at the 3' end.

The importance of methylation status of the pituitary POMC promoter in regulating POMC gene transcription was demonstrated by Newell-Price *et al* (Newell-Price et al., 2001). This promoter is embedded within a CpG island that is methylated in non-expressing tissues, but unmethylated in the pituitary and in ectopic ACTH secreting tumours. We examined the methylation status of the pituitary promoter in the GIST tumour by focussing on a specific portion -417 to -260 upstream of the transcription initiation site of exon 1. Hypomethylation at 3 CpG sites within this region were shown by Ye *et al* (Ye et al., 2005) to be highly specific for ACTH secretion in human pituitary and thymic carcinoids. Methylation analysis was performed as described (Chapter 2. Materials and methods) using pyrosequencing. Initial results suggest that the POMC pituitary promoter in the GIST is normally methylated. Additional positive and negative controls are required to validate this pyrosequencing approach. Even minimal methylation of the pituitary promoter at the sites described ought to prevent expression of the 1200nt POMC peptide, but would still allow the 800nt transcript to be expressed. However, as described above, this would result in a translated peptide lacking the signal sequence for ER targeting and subsequent secretion. The promoter responsible for initiation of proposed POMC expression in the GIST is therefore a key factor yet to be properly identified.

4.5.8 The steroidogenic activity of alpha-MSH

There is a long literature investigating the possible roles of α-MSH within the adrenal gland. Branchaud et al demonstrated corticotrophic activity of α-MSH in explants prepared from the cortex of the human fetal adrenal in 1978 at but only at concentrations of 10^{-6}M (Branchaud et al., 1978). Stimulation of aldosterone and corticosterone secretion by alpha-MSH in the rat was demonstrated in the 1980s by Vinson et al (Vinson et al., 1983; Vinson et al., 1981) and Baumann *et al* (Baumann et al., 1986). Robba *et al* described the effects of chronic alpha-MSH administration in causing zona glomerulosa hypertrophy and aldosterone secretion in the rat, via an action that required normal levels of circulating angiotensin II (Robba et al., 1986).

Our findings of alpha-MSH stimulation of cortisol production *in vitro* confirm the findings of Henville *et al* who demonstrated cortisol, aldosterone and corticosterone production from human adrenocortical cells in response to alpha-MSH and desacetyl-alpha-MSH (Henville et al., 1989; Branchaud et al., 1978). They found that the minimum effective concentrations of alpha-MSH was 0.1μM, whereas desacetyl-alpha-MSH was much more potent - minimum effective concentration 1nM. The concentration of alpha-MSH detected in GIST culture supernatant by ELISA was of the order of 1.0nM, which is therefore much less than the biological activity of the supernatant would predict. It is possible that additional desacetyl-alpha-MSH secretion from the GIST supplying some of this biological activity, while degradation of alpha-MSH in the supernatant used for EIA and ELISA assay may have led to an underestimate of the actual quantities, depending on the level of protease contamination in that system. A vulnerability of alpha-MSH to the thaw and refreeze cycle is suggested by the manner in which Sigma synthetic alpha-MSH failed to stimulate melanin production from mouse melanoma cells *in vitro*.

As mentioned previously, it is extremely unfortunate that we were unable to retrospectively assay the patient's serum for alpha-MSH following these experiments. The large volume of the GIST and the consistent immunostaining for alpha-MSH throughout it would be consistent with the possibility of serum levels several orders of magnitude higher than ACTH. The high concentration of alpha-MSH required to stimulate cortisol in our experiments as well as those quoted above (Henville et al., 1989; Branchaud et al., 1978), was several orders of magnitude above the levels of ACTH required to stimulate cortisol production *in vitro* from human adrenal cells, and 7 orders of magnitude higher

than that required by ACTH to stimulate cortisol production *in vivo*. This calls into question the relevance of this effect in physiological terms, although as noted above, the volume of the GIST is two orders of magnitude larger than the volume of the average normal human pituitary, so the levels of alpha-MSH in the circulation could potentially have been much higher than a normal serum ACTH level. Furthermore, it is not suggested that alpha-MSH is binding to the MC2R, as will be discussed in detail in chapter 5. As will be discussed, upregulation of melanocortin receptors upon repeated receptor binding could also serve to amplify the alpha-MSH signal over time (Xing et al., 2010).

Receptor binding studies using radio-labelled ACTH and alpha-MSH have demonstrated that the MC2R is very highly specific for ACTH alone, raising questions as to the identity of the adrenal alpha-MSH receptor (Schiöth et al., 1996). Doghman et al (Doghman et al., 2004; Doghman et al., 2007; Doghman et al., 2005; Dhillo et al., 2005)have identified an MC2R independent pathway for cortisol secretion in bovine adrenal cells stimulated by α-MSH in vitro via the melanocortin 4 receptor, and antagonised by agouti-related peptide (AGRP). AGRP is an agonist of alpha-MSH at the MC3R and MC4R receptors in the arcuate nucleus of the brain, and the same authors detected MC3R and MC4R in the rat adrenal gland. AGRP does not have significant antagonist activity at the MC2R, and does not antagonise the acute steroidogenic effect of ACTH, but is upregulated in Cushing's syndrome, suggesting an inhibitor paracrine role in the human adrenal (Dhillo et al., 2005).

4.5.9 Conclusion

We have described a case of ACTH-independent Cushing's syndrome in which it is proposed that alpha-MSH secretion from a GIST tumour provided a stimulus for adrenal cortisol production. This is supported by the stimulation of cortisol production in vitro from the supernatant from a primary culture of the GIST, and alpha-MSH immunostaining. Clinical evidence of skin pigmentation which resolved on removal of the GIST, and a reduction in size of the remaining left adrenal proved additional clinical evidence.

A steroidogenic effect of alpha-MSH has been extensively reported previously, although the precise identity of the adrenal receptor for this hormone is not yet known. This is the first report of hormone secretion from a GIST causing Cushing's, although there is one

previous instance of an alpha-MSH secreting tumour causing hypercortisolaemia in the absence of elevated ACTH - a melanotroph tumour of the pituitary gland in a cat (Meij et al., 2005).

Possible candidates for the adrenal alpha-MSH receptor will be discussed in Chapter 5, along with possible explanations for the pigmented appearance of the adrenal gland.

Chapter 5 The causes of adrenal pigmentation

5.1 Aims

Having concluded in the previous chapter that alpha-MSH secretion from the GIST was the stimulus for cortisol production from the patient's hyperplastic adrenal glands, this chapter seeks to determine what role this hormone had in the pigmentation of the patient's skin, and of the excised right adrenal. It is hypothesised that the pigment responsible is melanin in both cases. A mechanism will be proposed to account for this. This chapter further aims to extend this enquiry into the mechanism for pigmentation in pigmented adrenal diseases, and in normal adrenal physiology.

5.2 Introduction

Increased skin pigmentation in the context of adrenal disease is well-recognised. It is an important diagnostic sign in patients with adrenal insufficiency, and in patients presenting with ectopic ACTH secretion from a neuroendocrine tumour, both of whom have high levels of serum ACTH (Isidori et al., 2006; Addison, 1855). Both ACTH and α-MSH are ligands for the G-protein linked melanocortin-1 receptor (MC1R) on the melanocyte plasma membrane (Abdel-Malek et al., 1995; Suzuki, I. et al., 1996). Activation of a PKA-mediated signalling pathway up-regulates a number of enzymes involved in melanin production within the melanosome organelle (Slominski et al., 2004)(Figure 5.1). The entire organelle is then transferred directly from the melanocyte to the adjacent keratinocytes through numerous dendritic processes on the cell surface (Wu & Hammer, J. A., 2014). The process is activated by ultraviolet light, while production of ACTH and alpha-MSH within melanocytes and keratinocytes themselves is a key part of its local regulation of in normal skin.

Pigmentation within the adrenal gland is less well understood. Within the normal adrenal gland the presence of lipofuscin in the zona reticularis has been observed, although it has not been clear why the ZR alone should be the location for this pigment(Idelman, 1970). Lipofuscin is a highly oxidised cross-linked aggregate consisting of oxidised protein/lipid clusters (Jung et al., 2007). Its formation is hypothesised to start with oxidative damage

to protein structures within the cytosol, and mitochondrial oxidative stress, and as such it is considered a “wear and tear” pigment.

The most well-described adrenal pathology where increased levels of pigmentation have been observed is primary pigmented nodular adrenal disease (PPNAD). Most often seen as part of the Carney complex, PPNAD is characterised by a pigmented and micro-nodular adrenal, with Cushing's syndrome usually developing in childhood (Horvath, A. & Stratakis, 2007). As described previously, PPNAD is the result of a constitutively active PKA-mediated signalling pathway in adrenal cortex cells as a result of inactivating mutations in the regulatory subunit type 1a of PKA (PRKAR1A) (Kirschner et al., 2000) or in the phosphodiesterase enzymes PDE11A or PDE8B (Horvath, A. et al., 2006).

The aetiology of adrenal pigmentation in PPNAD has not been satisfactorily explained, but is most commonly attributed to lipofuscin deposition (Stratakis et al., 1999). However, this raises the question of why adrenal pigmentation is not a more prominent feature of adrenal glands in patients with other forms of ACTH-independent Cushing’s syndrome, and indeed in ACTH-dependent disease itself. The parallel mechanisms of PKA-mediated signalling pathways leading to elevated levels of cortisol in this disease, and the same processes leading to pigmentation in the skin suggest the appealing possibility that the same mechanism could apply in both locations.

This chapter considers the hypothesis that the major pigment involved in pigmented adrenal diseases is not lipofuscin but melanin.

5.3 Results

5.3.1 Adrenal pigmentation is due to the presence of melanin

The excised right adrenal contained multiple large, highly pigmented nodules. A brown pigmentation was seen on H&E staining. This pigment was eliminated by a pre-staining incubation with potassium permanganate to bleach endogenous melanin but not lipofuscin. The pigment stained black by the Masson-Fontana method, and did not stain pink with Ziehl-Neelsen or magenta with Periodic Acid Schiff (Figure 5.1). This provided strong evidence that the pigment was melanin rather than lipofuscin.

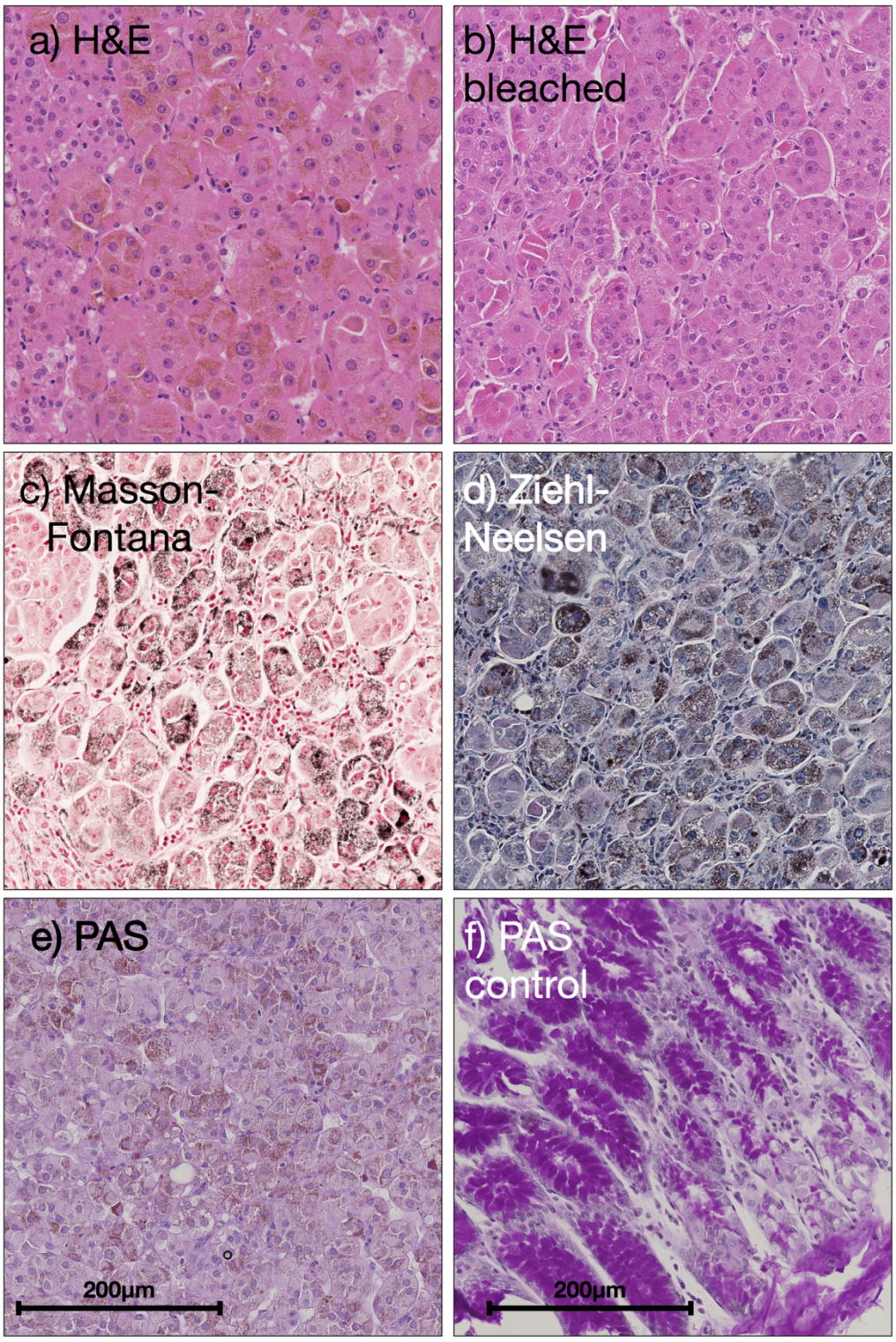

Figure 5.1 Adrenal pigmentation is due to the presence of melanin.
3µm sections of adrenal were subjected to a series of histological stains to ascertain the identity of a brown pigment visible within nodules on H&E (a). This is removed by pre-incubation with a potassium permanganate bleach that removes endogenous melanin but not lipofuscin. Masson-Fontana stains black with melanin. Ziehl-Neelsen stains lipofuscin pink, while PAS stains the glycolipid content of lipofuscin magenta. A positive PAS stain for bowel mucin is shown for comparison in (f)

Immunohistochemical staining was performed using a series of markers for melanocytic tissue - melan-A, S100, HMB-45 and MITF. The cortex of the whole adrenal gland exhibited strong staining for melan-A. There was mild staining for S100 within the pigmented regions of the adrenal and strong staining of the sustentacular cells of the adrenal medulla. HMB-45 and MITF staining was strongly positive within the cytoplasm of pigmented cells (Figure 5.2)

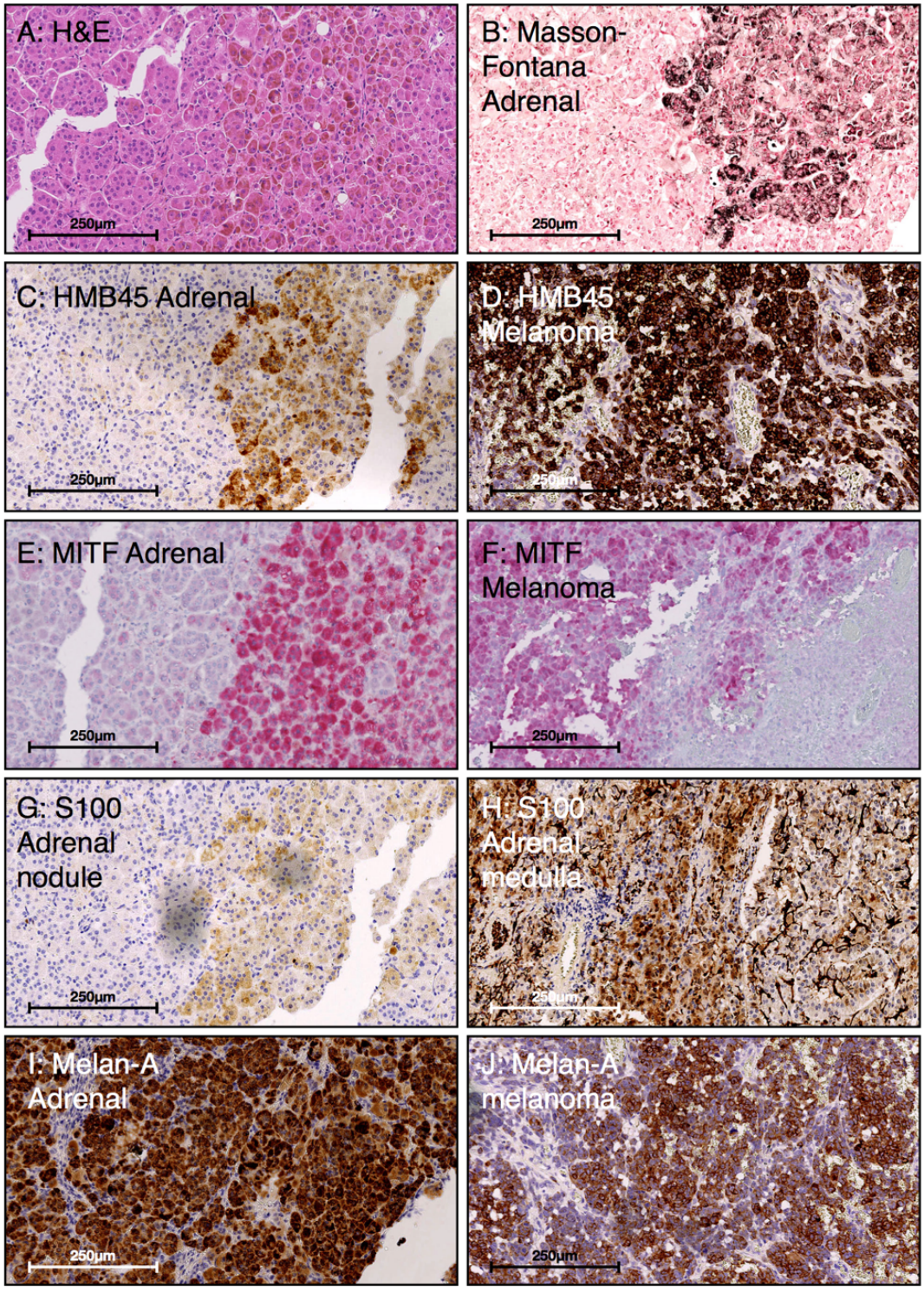

Figure 5.2 The pigmented adrenal gland expresses multiple markers of melanocytic cells
A series of immunohistochemical stains were performed on 3µm sections of pigmented adrenal gland. The pigmented nodules contain melanin (B – Masson Fontana), and stain positively for the melanosome marker HMB-45 (C), the marker of melanocyte signalling MITF (E), and the melanoma marker S100 (H). There is strong staining for Melan-A, but this marker is known to be widely expressed in adrenal cortex, although it is suggested that this is cross-reaction with an unknown epitope (see text). Full immunostaining method – Methods section 2.6.

Pigmented portions of the original gland were removed from paraffin blocks and processed for electron microscopy (EM). Scanning EM images of the pigmented portion of adrenal gland demonstrate multiple mature melanosomes throughout the cytoplasm, along with paler organelles which have some characteristics of lysosomes (Figure 5.3).

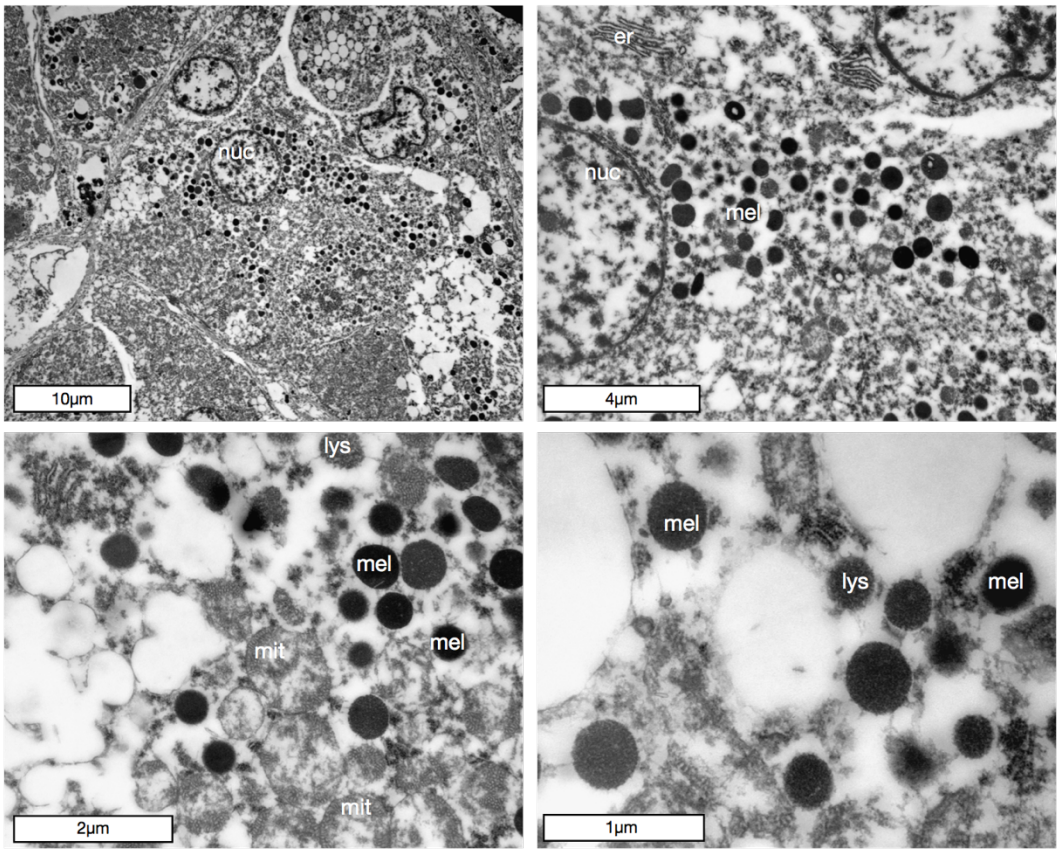

Figure 5.3 Pigmented adrenal gland reveals the presence of melanosomes under electron microscopy
Cores of pigmented adrenal from FFPE blocks were deparaffinised and tissues refixed in glutaraldehyde for processing ahead of electron microscopy. This leads to loss of intracellular lipid and some distortion of ultrastructure, but dark black mature melanosomes can be seen alongside paler organelles which may be lysosomes (lys) or may be immature melanosomes. Lipofuscin granules usually have a more irregular margin than the organelles seen here. Nuc – nucleus; mit – mitochondria; mel – melanosomes; lys – possible lysosome. Samples were visualised using a J.E.O.L JEM1230 transmission electron microscope and images captured with an Olympus "Morada" digital camera.

5.3.2 Human adrenal gland expresses the melanocortin 1 receptor

Having shown the presence of melanin within the adrenal gland, we hypothesized that alpha-MSH stimulation of the gland was causing this pigmentation. Since alpha-MSH is not thought to bind the MC2R we hypothesized that it may bind MC1R in the adrenal as

it does in skin (Schiöth et al., 1996; Rouzaud et al., 2005). RT-PCR was carried out on RNA extracted from the pigmented human adrenal gland, along with a commercially purchased human adrenal control RNA, and RNA from the human cell line THP-1 which has been shown to express the melanocortin-1 receptor (Taherzadeh et al., 1999). Expression of MC1R was demonstrated by this method in both control and patient (Figure 5.4).

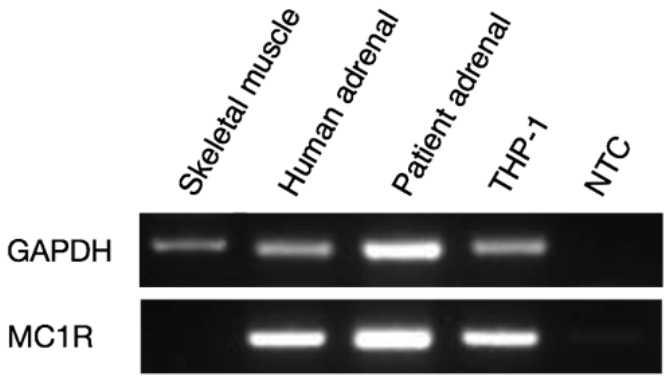

Figure 5.4 MC1R expression is demonstrated in human adrenal gland by RT-PCR
RNA was extracted from tissues using the RNeasy kit (QIAGEN) and RT-PCR carried out using primers designed against human melanocortin 1 receptor (see methods section 2.1). Skeletal muscle and control human adrenal cDNA was purchased from Clontech laboratories. THP-1 is a human cell line which has been shown to express MC1R. Both control human adrenal and the patient's adrenal in this case expressed MC1R.

The patient's MC1R gene was sequenced and found to contain the common polymorphism c.1831C>T (pArg151Cys) which is associated with red hair and an increased risk of melanoma(Raimondi et al., 2008).

The possibility that alpha-MSH binding to the MC1R was stimulating the parallel processes of cortisol production and melanin production was considered, and immunohistochemistry was performed on the pigmented human adrenal using antibodies directed against the MC1R and CYP11B1 - 11β-hydroxylase, the final enzyme in the cortisol synthesis pathway. Low levels of staining for both of these enzymes were present in the non-pigmented areas of the adrenal gland, with strong staining present in the pigmented areas (Figure 5.5).

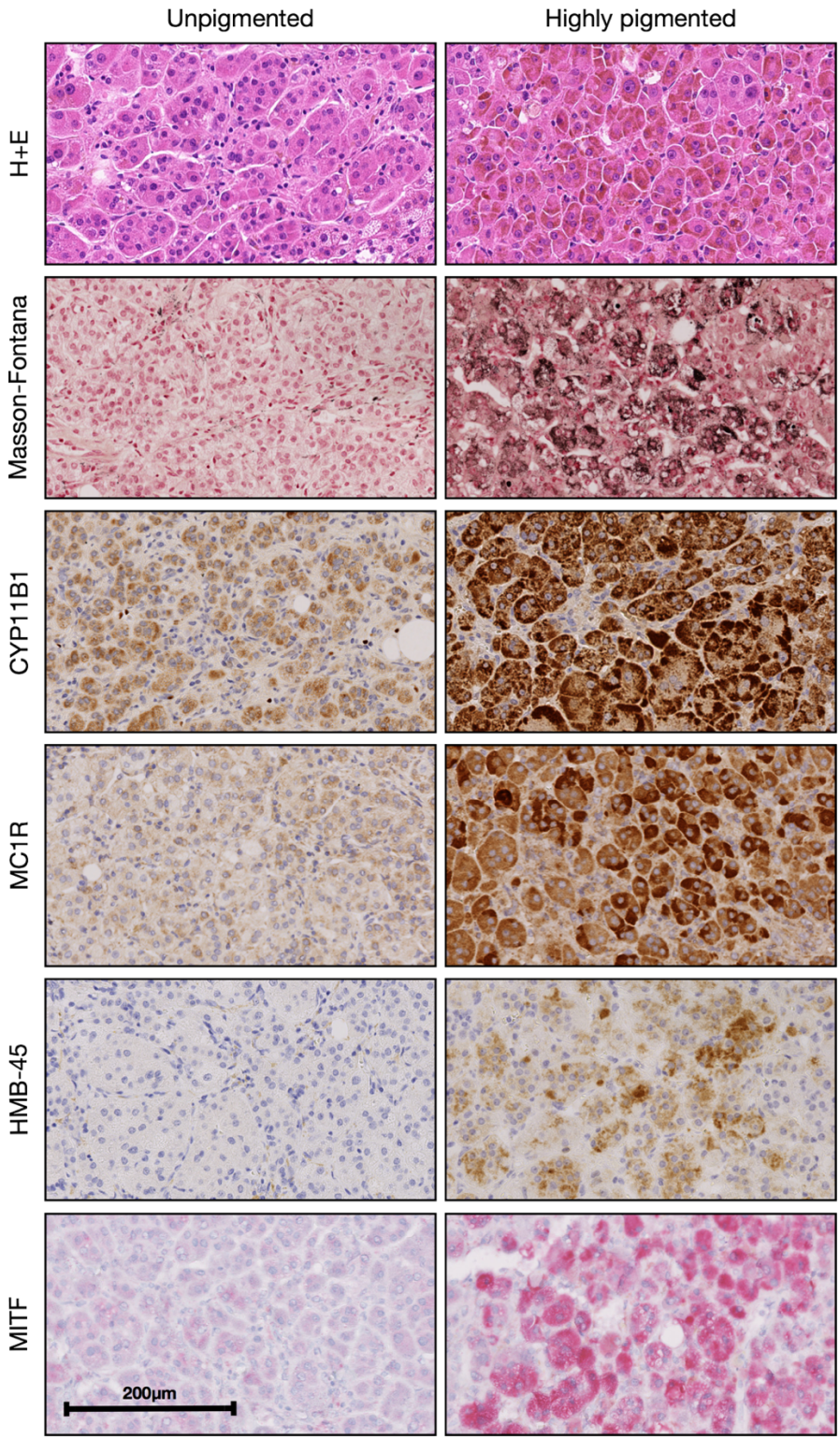

Figure 5.5 Pigmented adrenal demonstrates increased expression of the MC1R and 11β-hydroxylase

Sections of the patient's adrenal gland was immunostained for MC1R and CYP11B1, the final enzyme in cortisol synthesis (See methods section 2.6.1). The degree of staining was high for both antibodies within the pigmented portions of the gland, and only mild outside these areas. Melanin staining with Masson-Fontana and the melanocytic markers HMB45 and MITF stain in the pigmented areas as already described

5.3.3 Adrenal nodules in primary pigmented nodular adrenal disease contain melanin, and immunostain for markers of the melanosome organelle.

Consent was taken from 3 patients who had previously undergone bilateral adrenalectomy for Cushing's syndrome as a result of PPNAD. In all three cases PPNAD was an isolated finding, and there were no other signs of Carney Complex. Formal genetic testing had not yet been performed. All three patients were consented at the time of surgery for their pathological specimens to be used for research, and consented again for the specific experiments described below. Patient A is a female patient who was 11 years old at the time of diagnosis and adrenalectomy. Patients B and C were males who were 17 and 13 years old respectively. In all three cases H&E staining demonstrated multiple non-encapsulated cortical nodules composed of eosinophilic cells, most of which contained variable amounts of pigment. Formal pathology reports of the time did not describe any attempts to classify the pigment.

Sections from each patient were stained for H&E, Masson-Fontana for melanin, and immunostained for the melanocytic markers HMB-45 and (Figure 5.6). Immunostaining was also carried out for the presence of CYP11B1 and the MC1R. The nodules were shown to contain variable amounts of pigment on H&E staining, but in all cases this was positive by Masson-Fontana, identifying it as melanin. Counterstaining with 1% neutral red was less intense than on previous Masson-Fontana stains. Staining within the nodules was strong for CYP11B1 and MITF and moderate for HMB45 and MC1R, while there was minimal staining outside the nodules.

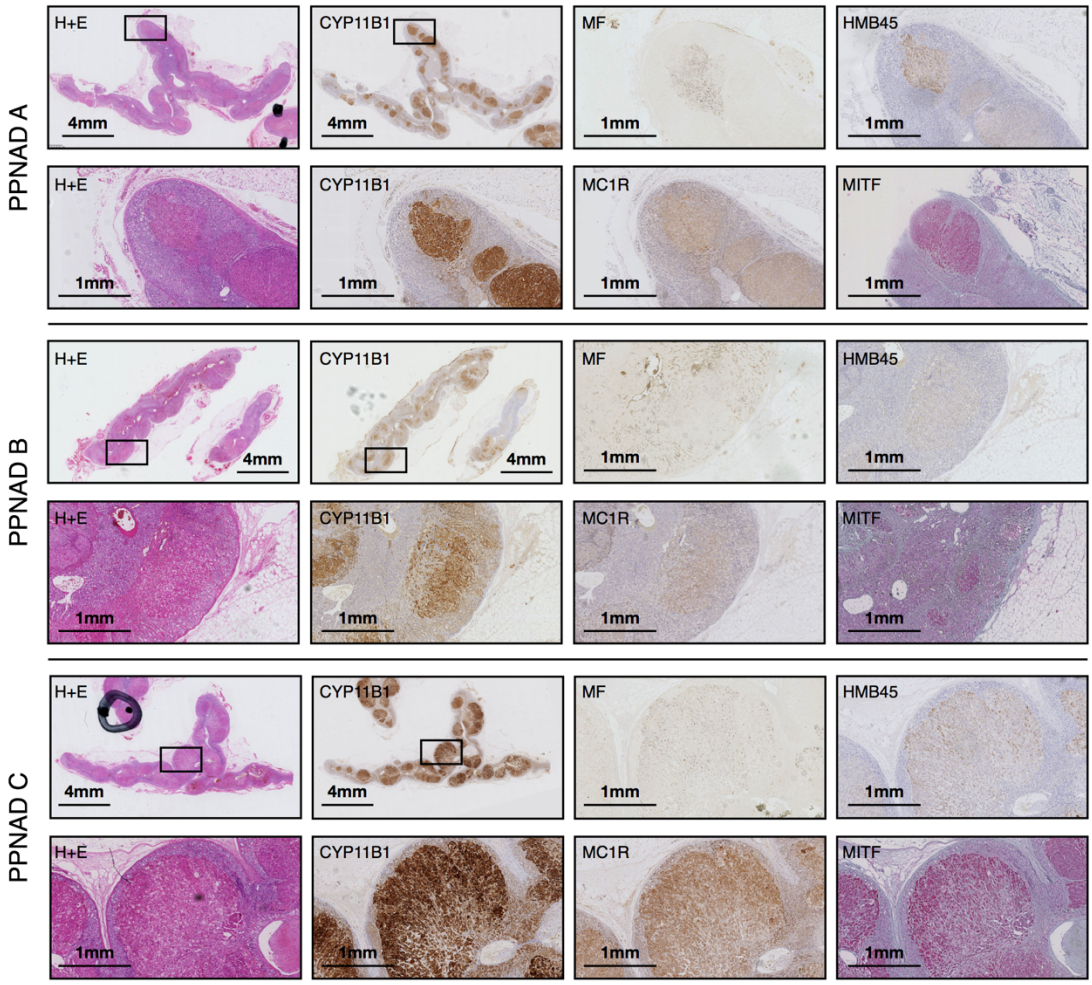

Figure 5.6 Adrenal nodules in PPNAD contain melanin and stain for the melanocytic markers HMB45 and MITF
Adrenal glands from three patients with ACTH-independent Cushing's syndrome as a result of PPNAD were immunostained for MC1R, CYP11B1, HMB45 and MITF (For full method see section 2.6.1). Nodules contained melanin in all three cases, and stained positively for all 4 antibodies. Masson-Fontana staining was performed without red counterstaining.

5.3.4 The zona reticularis of adrenal glands from patients with ACTH-dependent Cushing's contains melanin, and immunostains positively for markers of the melanosome organelle.

Consent was taken from three patients with ACTH-dependent Cushing's syndrome for histological analysis of their adrenal glands. Patient A was a 34-year-old male at the time of left adrenalectomy. He had been unsuccessfully treated for Cushing's disease with transsphenoidal hypophysectomy at 27, and had received external beam radiotherapy to the pituitary at 29, but remained biochemically and clinically Cushingoid. He underwent a left adrenalectomy to reduce the burden of cortisol secretion. Patient B was a 39-year-

old male who had ACTH-dependent Cushing's syndrome from an occult ectopic source who was cured of Cushing's with bilateral adrenalectomy. Patient C had a metastatic ACTH-secreting neuroendocrine tumour who was treated briefly with intravenous etomidate before an emergency bilateral adrenalectomy.

Histological and immunohistochemical staining was carried out as before. In all three cases the adrenal cortex immunostained positively for MC1R and CYP11B1. The presence of melanin was detected in the zona reticularis, along with varying degrees of positivity for HMB45 and MITF (Figure 5.7). The zona reticularis was expanded in patients A and C.

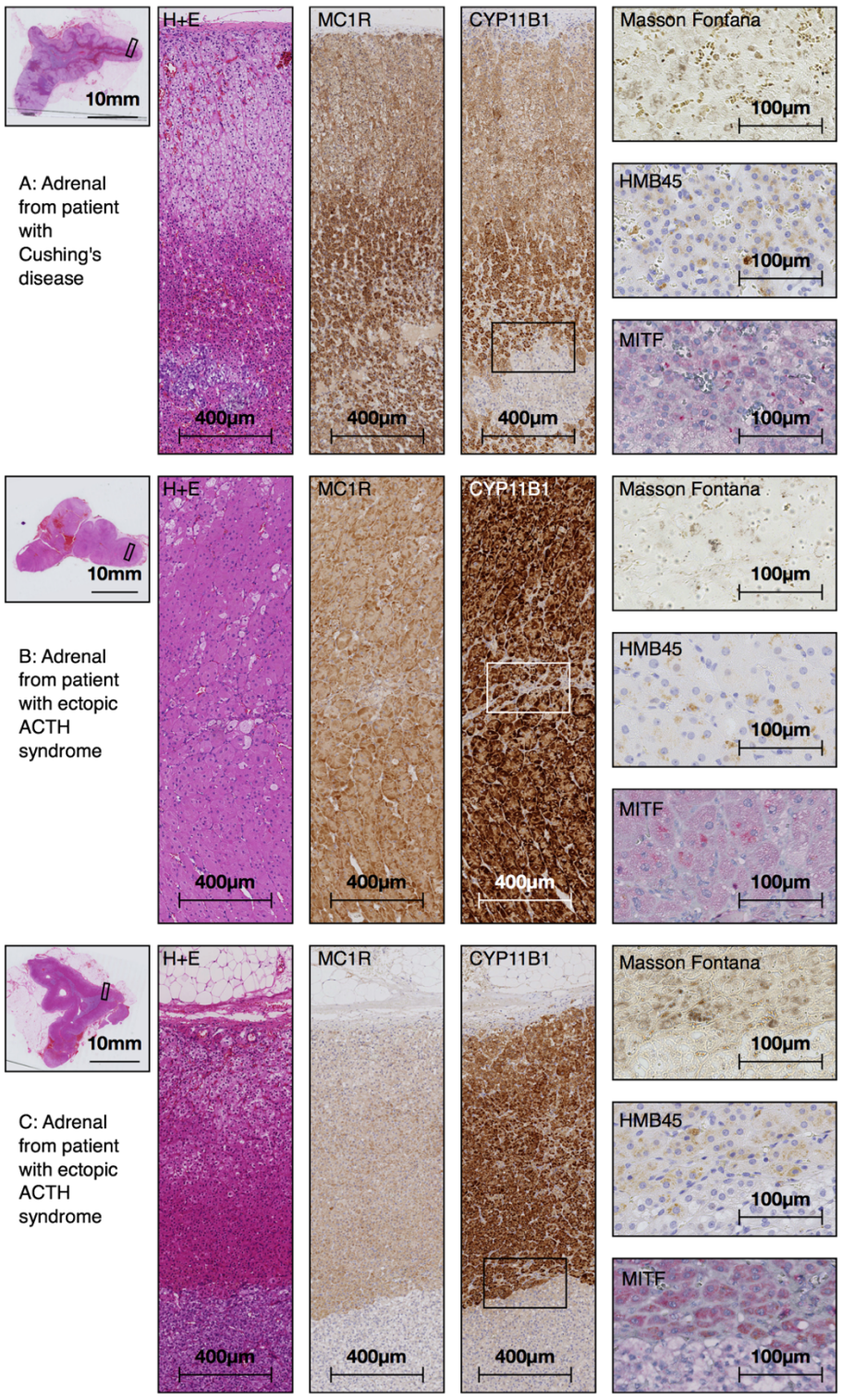

Figure 5.7 Adrenal glands from patients with ACTH-dependent Cushing's syndrome contained melanin in the ZR, and immunostained for melanocytic markers

Adrenal glands from three patients with ACTH-dependent Cushing's were immunostained for MC1R, CYP11B1, HMB45 and MITF. In all three cases melanin was detected in the zona reticularis (ZR) by Masson-Fontana staining, along with the melanosome marker HMB-45 and the melanocyte signalling marker MITF. Two of the patients (A and C) demonstrated significant ZR expansion.

5.3.5 The zona reticularis of normal adrenal cortex from patients with primary aldosteronism expresses the melanosome markers HMB45 and MITF

A tissue microarray (TMA) was constructed from adrenalectomy specimens from patients with biochemically confirmed Conn's syndrome, which had been radiologically confirmed with a combination of CT scanning and bilateral adrenal venous sampling were required. All samples contained a single adenoma. The TMA was stained for H&E, and immunostained for HMB45 and MITF. "Normal" cores from 15 glands were reviewed.

14 glands demonstrated cytoplasmic staining for HMB45 and MITF within tissue that had been identified as zona reticularis and outside the nodule by a consultant histopathologist. The MITF and HMB45 staining correlated in each case, and in a number of cases obvious pigmentation could be seen on H&E staining. (Figure 5.8).

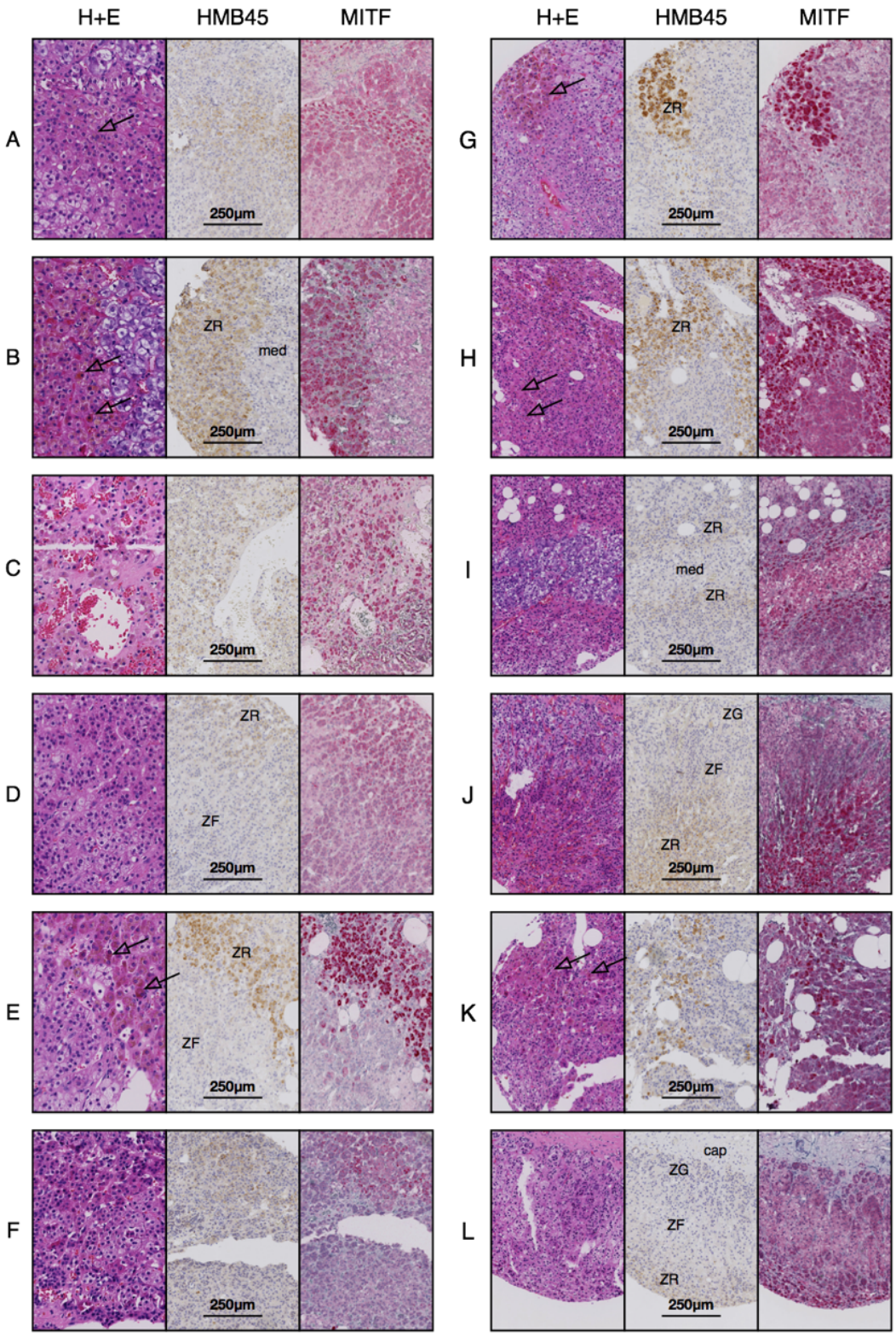

Figure 5.8 Normal adrenal tissue stains positively for melanocytic markers within the ZR
A tissue microarray was constructed using adrenal nodules and extra-nodular tissue from patients with Conns syndrome (see methods section 2.5.8) TMA cores were analysed from 15 patients having adrenalectomy for Conn's syndrome. Tissue identified as normal by a histopathologist are shown, staining positively for HMB45 and MITF within the ZR.

In addition to this cytoplasmic staining, 7 specimens demonstrated nuclear staining for HMB45 alone in adrenal cortex that had the morphological appearance of ZG or ZF. Functional staining to clarify the identity of these cells using CYP11B2 was not available.

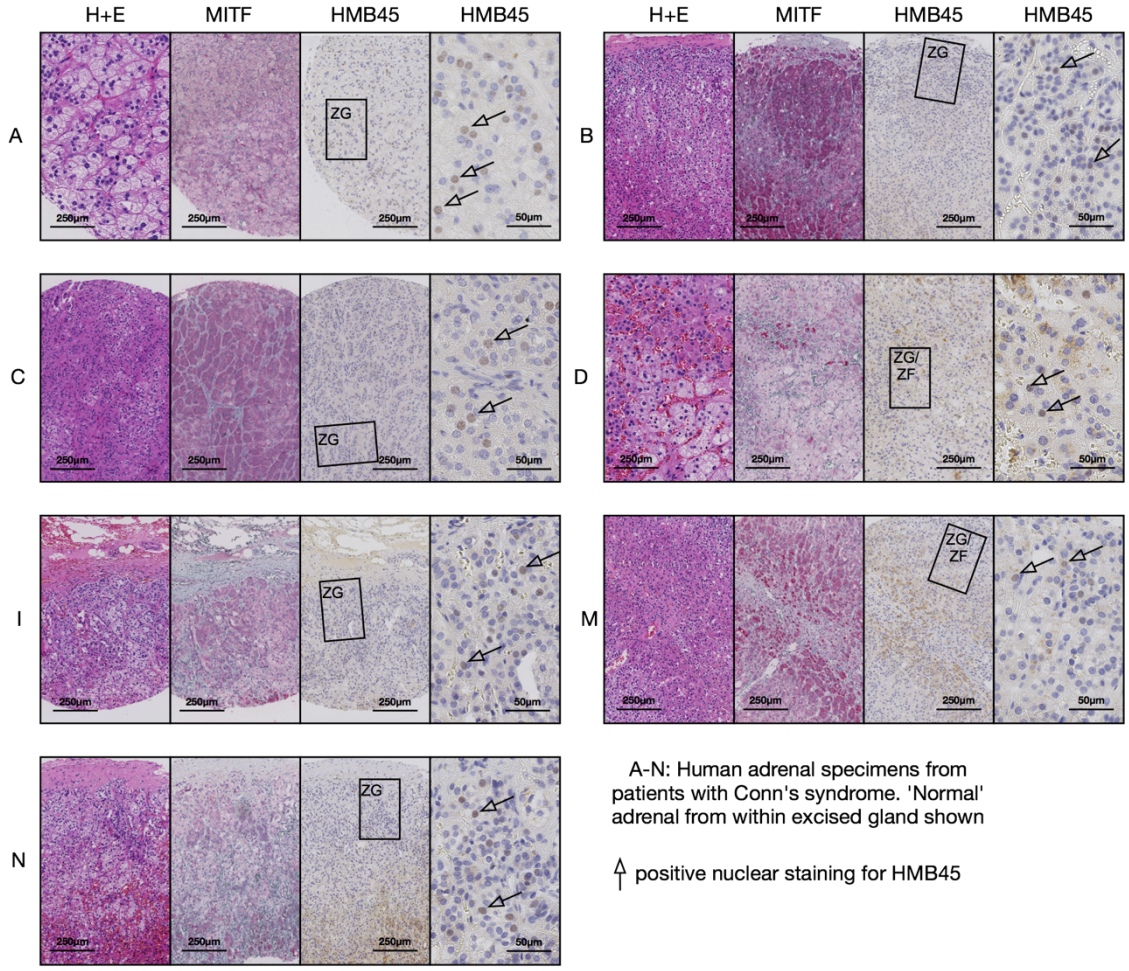

Figure 5.9 Nuclear HMB45 staining is seen within ZG and ZG cells of normal human adrenal
Extra-nodular tissue from human adrenal tissue taken from patients with Conns syndrome was immunostained for MITF and HMB45 on sections from the Conns TMA. Moderate nuclear staining for HMB45 was demonstrated within ZG and ZF cells of normal extra-nodular adrenal.

5.4 Discussion

5.4.1 Lipofuscin

As outlined previously, the most likely cause of adrenal pigmentation is lipofuscin, on the basis of previous descriptions of this pigment within the zona reticularis. However, the

presence of melanin has previously been described within the chromaffin cells of the adrenal medulla, and pigmented phaeochromocytomas have been reported in the literature (Bellezza et al., 2004; Handa et al., 2005). In the light of skin hyperpigmentation, and the presence of alpha-MSH within the GIST tumour, a hypothesis was made that the adrenal pigmentation was the result of melanin within the adrenal cortex. It is worthwhile considering these two pigments prior to describing their histological diagnosis.

Lipofuscin is a highly oxidised cross-linked aggregate consisting of oxidised protein/lipid clusters (Jung et al., 2007). The formation of lipofuscin is a complex process which is hypothesised to start with oxidative damage to protein structures within the cytosol, or with mitochondrial oxidative stress. Failure of the normal intracellular repair mechanism results in the proteins or mitochondria becoming cross-linked, then taken up by lysosomes. They are processed further and accumulate more damaged material, leading to lysosomal rupture, with lipofuscin released into the cytosol to do more damage and continue the cycle. The ability of lipofuscin to bind metals is key to this cellular toxicity (Jolly et al., 1995). Lipofuscin deposition and its toxic effects are felt more keenly in post mitotic cells such as cardiac myocytes and neutrons - the “Lipofuscin Hypothesis of Alzheimer’s disease” describes the theory that it has a role in the formation of senile plaques in that disease (Giaccone et al., 2011)

The reason for the presence of lipofuscin in the zona reticularis of the adrenal cortex but not the zona fasciculata has not been satisfactory explained. One view follows from the classical theory of adrenal donation driven by cell migration from a common pool of progenitors in the peripheral cortex, suggesting that the ZR should be considered a zone of cell senescence, prior to apoptosis removing these cells from the system (Hui et al., 2009). Interestingly, it has also been suggested that oxidation of melanin can directly lead to the accumulation of lipofuscin (Kayatz et al., 2001). Granules of “melano-lipofuscin” have been implicated in the pathogenesis of age-related macular degeneration (Mazzitello et al., 2009).

Lipofuscin and melanin are indistinguishable on H+E staining, so a number of special stains are required. Lipofuscin is an insoluble pigment which is not removed by the potassium permanganate bleaching of histological specimens which removes melanin

(Hongwu Shen, 2015). Lipofuscin is a deep magenta red on Ziehl-Neelsen staining and magenta on PAS staining (Dayan et al., 1988). By all three of these criteria the adrenal pigment was not consistent with lipofuscin.

5.4.2 Melanins

Melanins are the end products of complex multistep transformations of L-tyrosine, and include eumelanin, phaeomelanin and neuromelanin. L-DOPA is a precursor to both melanins and catecholamines via separate pathways, but the catecholamines can also be converted into melanin pigments. Synthesis of the red/brown phaeomelanins is the default pathway, whereas the black eumelanins require higher levels of tyrosine and activity of the melanogenic enzymes tyrosinase and tyrosinase-related proteins TRP1 and TRP2 (Abdel-Malek et al., 2008). MC1R mutations which reduce the efficacy of MC1R signalling are therefore associated with reduced eumelanin production, and therefore the typical appearance of red hair and pale skin (Switonski et al., 2013). The patient studied had one such mutation, and it would be expected that the effect on MC1R signalling would be just as limited in the adrenal gland as in the skin. However, the effect of the MC1R polymorphism results in reduced activity, rather than zero activity, so high levels of stimulation of adrenal MC1R in this patient from alpha-MSH over a prolonged period of time would still be expected to cause activity. Additionally, this would also be anticipated to lead to receptor upregulation, and therefore amplify the signal (Xing et al., 2010). Furthermore, the possibility remains that one or more of the adrenal nodules was displaying a degree of autonomy, further elevating the effects of alpha-MSH.

The adrenal nodules demonstrated positive staining by the Masson-Fontana method for melanin, and the pigment was removed by bleaching.

5.4.3 Melanoma immunohistochemistry

A series of immunohistochemical stains which are more commonly used in the diagnosis of melanoma were performed to further characterise the pigment.

Melan-A (also known as MART-1) is a melanoma associated antigen which is present in melanosomes and the ER and plays a role in the expression and processing of pmel17 (recognised by HMB-45) (Coulie et al., 1994). It is highly sensitive for melanoma, but is

also expressed in more than 80% of adrenocortical tumours, and in steroidogenic tissue in the testis (Sangoi et al., 2011; Renshaw & Granter, 1998; Zhang et al., 2003). It has been suggested that in fact positivity for Melan-A in the adrenal cortex is the result of cross-reactivity with an unidentified epitope, and that the Melan-A gene is not expressed there (Busam et al., 1998) Melan-A positivity was present throughout the cortex of the adrenal gland in this case.

S100 was the first marker to prove useful in the diagnosis of melanoma (Gaynor et al., 1980). It has a sensitivity of 93-100% in this circumstance, but its specificity is low due to its expression in a wide variety of normal tissues (adipocytes, chondrocytes, certain neuronal populations) and other tumours (breast and salivary gland tumours, some sarcomas) (Ordóñez, 2014); this is a consequence of the common neural crest derivations of these tissues. As demonstrated in figure 5.2 it is highly expressed in the sustentacular cells of the adrenal medulla (Lloyd et al., 1985) and is used as a histological marker for phaeochromocytoma for that reason. The adrenal cortex has not been shown to express S100 to this point. S100 staining was strong as expected in the adrenal medulla, with mild staining of pigmented regions of the cortex. The protein is structurally similar to calmodulin, and has no specific mechanistic link to the presence of melanin or the melanosome.

HMB-45 is one of several monoclonal antibodies generated by Gown *et al* to an extract of melanoma (Gown et al., 1986). The HMB designation comes from a description of this extract: human melanoma black. The antigen target Pmel17 (gp100 in the mouse) is involved in the morphogenesis of immature melanosomes, and is such demonstrates cytoplasmic staining (Kikuchi et al., 1996; Berson et al., 2001). HMB-45 is therefore regarded as being specific for the melanosome, rather than any particular cell lineage. In addition to melanocytes and melanoma, positivity can also be seen in perivascular epithelioid cell tumours (PEComa) (Doyle et al., 2013; Patra et al., 2013), and various pigmented neuroectodermal tumours, such as the melanotic schwannoma (Utiger & Headington, 1993; Leger et al., 1996). Interestingly, the latter is a component of the Carney complex, although a less common one than PPNAD. There was moderately positive immunostaining for HMB-45 within the pigmented adrenal tissue in this case, suggesting the presence of the melanosome organelle.

MITF (micro-ophthalmia associated transcription factor) is the master regulator of melanocytes, and plays a key role in their development, differentiation, function and survival (Hsiao & Fisher, 2014). The role of the mouse equivalent Mitf was first identified in 1979 when mouse with semi-dominant mutations showed a loss of pigmentation, reduced eye size, failure of secondary bone resorption and early onset deafness (Silvers, 1979). The human MITF gene has been mapped to chromosome 3p12.3–14.1 (Udono et al., 2000), and mutations have been associated with various combinations of abnormal skin pigmentation and sensorineural deafness, for example Waardenburg syndrome and Tietz syndrome.

Transcriptional control of MITF is governed by a number of transcription factors, with CREB (cAMP response element binding protein) and β-catenin of particular importance. Both these factors participate in the upregulation of MITF in response to α-MSH binding to the MC1R on the melanocyte cell membrane (Hartman & Czyz, 2015). Targets of MITF include regulators of the cell cycle and differentiation such as CDK2 and p21kip1, and enzymes necessary for melanin synthesis including tyrosinase (Hsiao & Fisher, 2014)

MITF dependence is retained in melanoma, where expression at high levels favours differentiation or proliferation. MITF is recognised as a sensitive and specific marker for melanoma diagnosis (King, R. et al., 1999), where a strong nuclear staining pattern is apparent with immunohistochemistry (Sheffield et al., 2002). However, cytoplasmic staining for MITF has been shown in a small number of breast carcinomas (Granter et al., 2002; King, R. et al., 1999), and the subcellular localisation of MITF has been shown to be dynamic in monocytic cells (Lu et al., 2010). Immunohistochemical staining of the adrenal cortex for MITF has not been demonstrated previously, although transfection of H295R cells with MITF has been shown to significantly increase their cortisol secretion(Romero et al., 2010). There was strong MITF immunostaining in the pigmented adrenal cortex of this case.

5.4.4 Electron microscopy

The immunohistochemical staining of the adrenal nodules supported the hypothesis that the pigmentation was melanin within melanosomes. Electron microscopy (EM) was carried out to visualise the melanosomes directly. Processing of tissue for EM is best performed on fresh tissue, by fixing immediately in 2.5% glutaraldehyde. Tissue can

however be extracted from FFPE tissues, although some structural integrity as lost. Despite this caveat, substantial numbers of mature, dark round and oval melanosomes were visualised in the pigmented tissue from one of the large adrenal nodules. Melanosomes can be distinguished from lipofuscin within lysosomal granules on the basis of their more regular shape, although some of the less dark oval bodies within the EM images shown have the appearance of lysosomal granules.

5.4.5 Control of melanin synthesis

Having established the presence of melanin and melanosomes in pigmented adrenal cortex, we hypothesised that this had been stimulated by the binding of alpha-MSH to the MC1R. Binding of this receptor in the melanocyte by alpha-MSH or ACTH stimulates cAMP formation (Abdel-Malek et al., 1995). Activation of the PKA signalling pathway results in phosphorylation of CREB, and increased MITF expression (Figure 5.10). This in turn regulates the expression of multiple enzymes in the melanin biosynthetic pathways, including tyrosinase, and in melanosome maturation (Tsatmali et al., 2002). Sites of alpha-MSH production in the skin include epidermal keratinocytes, Langerhans cells, and melanocytes themselves. Ultraviolet radiation stimulates the process of pigmentation by increasing the expression of POMC, the MC1R itself, and melanogenic enzymes such as tyrosinase directly (Abdel-Malek et al., 2008).

Expression of the MC1R in human adrenal was confirmed by RT-PCR, and by immunohistochemistry. Unfortunately, we were unable to optimise immunohistochemistry for the MC2R in the adrenal. This raises the possibility that the staining demonstrated within the adrenal is in fact MC2R, however the peptide sequence used to raise the MC1R antibody is not present in MC2R, and a basic local alignment search tool (BLAST) search suggested no similarity, so we are confident that we have not demonstrated MC2R expression with this antibody. However, further evidence could be provided by adding an incubation with MC2R peptide to the MC1R staining protocol. MC1R expression was most pronounced within the pigmented regions, and up regulation of melanocortin receptors in response to chronically activated cAMP signalling is well described (Xing et al., 2010).

Upregulated cAMP signalling would be expected to lead to increased expression of steroidogenic enzymes within the adrenal cortex, and we were able to show increased

CYP11B1 expression which correlated with pigmentation, immunohistochemical staining for the melanosome, and MC1R expression. This suggests that pigmentation and cortisol production are being activated in parallel by a common signalling pathway.

5.4.6 Primary pigmented nodular adrenal disease

Having demonstrated parallel increases in steroidogenic enzyme expression and pigmentation, we examined three cases of PPNAD to establish whether a similar mechanism could be responsible adrenal pigmentation and Cushing's syndrome in that condition. The rarity of the condition meant that availability of archived samples was restricted to three adrenalectomy specimens, and unfortunately we did not have access to full sufficient data to correlate pathological appearances with the clinical and biochemical phenotype. Melanin was detected within PPNAD micro nodules in all cases, along with MC1R, CYP11B1, HMB45 and MITF immunohistochemistry. This suggests that there is a common mechanism is contributing to both processes, as in the GIST-associated Cushing's syndrome case. If we suggest that in the GIST case, exogenous stimulation of MC1R by alpha-MSH or related peptides activates cAMP signalling with melanosome maturation, melanin production and unregulated steroidogenic enzyme expression then by extension we might predict that the same outcome could be generated by PRKR1A or PDE11A mutations in PPNAD.

The nodular enlargement of both PPNAD and the GIST-associated adrenal, rather than a diffuse hyperplasia, is a common feature of endocrine neoplasia. The specific genetic and epigenetic differences within the nodules in adrenals such as those described, or in multi nodular thyroid goitre, for instance, is not well understood(Derwahl & Studer, 2002).

It is interesting to note that the staining characteristics that we have demonstrated in PPNAD have also been shown in extra-adrenal pigmented tumours associated with Carney complex. Psammomatous melanotic schwannomas positively immunostain for Melan-A, S100 and HMB-45, and demonstrate melanosomes on electron microscopy (Utiger & Headington, 1993; Leger et al., 1996; Torres-Mora et al., 2014).

5.4.7 ACTH-dependent Cushing's syndrome

We assessed adrenal glands from three cases of Cushing's syndrome as a result of ACTH secretion - one pituitary dependent, two from ectopic sources. These glands exhibited a

diffuse hyperplasia, with two of the three showing an expansion of the zona reticularis. In all three cases we were able to identify melanin by Masson-Fontana staining, and immunohistochemical staining for HMB-45 and MITF. MC1R and CYP11B1 staining was strongest within the ZR, although it is possible that this reflects simply the different cellular structures: increased lipid in the clear cells of the ZF compared with the compact, dense cells of the ZR. The ZR expansion is consistent with the previously described change in morphology of ZF cells under chronic ACTH stimulation (Hornsby, 2002).

5.4.8 Pigmentation within normal adrenal gland

It is difficult to obtain entirely normal human adrenal tissue for study, with the most common means of doing so adrenal glands which are removed during nephrectomy for non-endocrine diseases. Previous studies on the pathophysiology of primary aldosteronism led us to construct a tissue microarray from tumour specimens and "normal" adrenal tissue taken from within the same gland. Whether this tissue can actually be thought of as normal is not entirely clear, as it has been reported that tissue outside the nodule can show evidence of hyperplasia. This is consistent with the notion that there is a spectrum of abnormality between hyperplasia and adenoma. Notwithstanding this, on 14 of 15 adrenal glands reviewed we identified zona reticularis with positive immunostaining for HMB45 and MITF. The presence of these markers would not therefore seem to depend on the presence of Cushing's syndrome, either from exogenous stimulus of the gland or cortisol secreting adenoma as with PPNAD. None of the Conns samples studied had any evidence of hypercortisolaemia clinically or biochemically.

In addition to this cytoplasmic staining, we were also able to demonstrate nuclear staining for HMB45. It would be useful to identify with more certainty the additional characteristics in terms of functional steroidogenic gene expression of these cells. Functional staining of CYP11B2 would be extremely valuable to confirm the nature of these cells as ZG, but as discussed in chapter 3 immunohistochemistry for this enzyme is not straightforward, and it was not available to us at the time of staining. Nuclear staining for HMB45 has recently been used to discriminate between benign and malignant melanocytic lesions, with a higher ratio of nuclear: cytoplasmic staining in benign lesions. Melanosome formation begins at the perinuclear region, so nuclear staining may

represent cells in which pathways for melanosome formation have not yet been fully activated.

Melanosome development proceeds through a series of defined stages (Seiji et al., 1963). Stage I premelanosomes contain internal membranous vesicles that resemble late endosomal multivesicular bodies, and irregular fibrous structures, but they do not contain any pigment. They were originally thought to have emerged from the smooth ER, but are now believed to be derived from the late secretory or endosomal pathway (Raposo et al., 2001). These become arranged in regular parallel rows in type II premelanosomes, with a striated appearance on electron microscopy. Melanosomes acquire the tyrosinase enzyme and melanin pigments are deposited on these fibres as they mature into darker stage III and IV melanosomes (Tsatmali et al., 2002). The glycoprotein Pmel17 (which stains with HMB45) is a key mediatory of this fibrillary matrix (Berson et al., 2001).

In melanocytes fully formed melanosomes are transferred into neighbouring keratinocytes and leave the cell. In cells where melanosome transfer is not possible, it might be predicted that younger cells would have the fewest melanosomes, and older cells the most. In the adrenal gland, this would be in keeping with the centripetal migration hypothesis described earlier. Immunofluorescent staining for HMB45 would be extremely informative in this regard.

5.4.9 Possible functional role of melanin in adrenal tissue

These data raise the question of what might be the role of melanin in the adrenal gland. It is instructive here to consider what its role is in tissues outside of the skin.

Neuromelanins are a group of melanin pigments found in the central nervous system. They include substantia nigra melanin and locus coeruleus melanin which are formed by the oxidation of catecholamines(Wakamatsu et al., 2015). The role of these neuromelanins is believed to be in protecting neurons against oxidative stress. For instance, melanin in the inner ear is believed to protect the organ of Corti from noise and ototoxins (Ohlemiller et al., 2009). Chromaffin cells have the same neural crest embryological origin as melanocytes, and melanin-containing phaeochromocytomas have been described (Bellezza et al., 2004; Handa et al., 2005). We found no pigment or melanosome staining present in the adrenal medulla, but we are open to the possibility that the melanin pigment in the ZR is neuromelanin. Direct melanosome transfer from

chromaffin cell to ZR would be in keeping with the model of melanosome transfer in the skin, with ZR cells equivalent to keratinocytes. However, this would not explain the degree of MITF expression in those cells.

The retinal pigmented epithelium (RPE) is a monolayer of pigmented cells forming part of the blood/retina barrier, interposed between the neural retina and the choroid (Fuhrmann et al., 2014). It plays a key role in the absorption of scattered light energy, and protecting the retina from photo-oxidative damage: light absorption via melanin in melanosomes is one of the major mechanisms for this (Strauss, 2005).

The anti-oxidative properties of melanin pigments are not only manifest in the nervous system. Randhawa et al demonstrated the expression of several melanogenesis related genes in human adipose tissues, and subsequently confirmed the presence of melanin itself. They proposed that ectopic synthesis of melanin served as a compensatory mechanism utilising its anti-inflammatory and oxidative damage-absorbing properties. (Randhawa et al., 2009). In addition to this, significantly elevated levels of α-MSH have been found in the sera of obese human subjects, perhaps as a homeostatic anti-inflammatory mechanism (Page, S. et al., 2011).

It may be hypothesised that the anti-oxidant properties of melanin may be valuable within the adrenal gland. Electron leakage by the cytochrome p450 enzymes may render the adrenal cortex vulnerable to oxidative stress, and this is implicated in adrenal disorders including X-linked adrenoleukodystrophy, triple A syndrome and familial glucocorticoid deficiency (Prasad et al., 2014). It has been suggested that oxidation of melanin can directly lead to the accumulation of lipofuscin (Kayatz et al., 2001). The presence of the melanolipofuscin granule which exhibits properties of both pigments, and is implicated in the pathogenesis of age-related macular degeneration would support this hypothesis (Mazzitello et al., 2009).

5.4.10 Conclusion

In conclusion we report the finding of a pigmented macronodular adrenal gland in a patient with a co-existing GIST which immunostains positively for alpha-MSH. The pigmented nodules contain melanin and positively immunostain for markers of the melanosome organelle. The alpha-MSH receptor MC1R is present and upregulated in

pigmented tissue, along with additional markers of increased cAMP signalling: the steroidogenic enzyme CYP11B1 and the melanocyte transcription factor MITF. We suggest that upregulation of the cAMP dependent PKA signalling pathway in PPNAD is directly responsible for stimulating the parallel processes of steroidogenesis and melanogenesis within adrenal nodules. The process of melanogenesis is proposed to be a feature of normal adrenal tissue, particularly in the zona reticularis, and a function as an antioxidant is hypothesised.

Chapter 6 General Discussion

The panoply of physiological roles held by melanocortin peptides signalling in multiple different physiological systems has become increasingly apparent over recent years. The importance of interdisciplinary communication and collaborations to expand and build on this knowledge is vitally important, otherwise a dermatologist may simply focus on the role of the MC1R in melanoma, a rheumatologist on melanocortin signalling in inflammation, and an endocrinologist may restrict their focus to the MC2R in the adrenal cortex. The initial focus in beginning this work was on the particular role of the MC2R in adrenal development, building on observations that adrenal-specific knockout of the MC2R in the mouse led to embryonic lethality in 75%, and that surviving animals had an atrophied ZF and a thickened capsule (Chida et al., 2007). A failure to obtain the KO mouse model meant that we were unable to study the receptor in that context, but the mice who survived without an adrenal MC2R suggest that alternative signalling mechanisms can perform some of the roles that we normally associated with it, in terms of steroidogenesis and adrenal growth.

It has been noted already (See Introduction) that the number of inputs to the adrenal gland that modulate steroid output is substantial, from cytokine production from immune cells within the gland, the effect of autonomic nervous inputs from the hypothalamus and contralateral adrenal, to neuropeptides secreted by the adrenal medulla. These additional levels of control allow the adrenal gland to respond to changes in the physiological milieu in a subtler and more controlled way than a simple ATCH to MC2R on/off switch signalling mechanism would allow. It is therefore not surprising that the adrenal gland would contain other melanocortin receptors sharing an input into the same downstream signalling pathways (Doghman et al., 2004; Doghman et al., 2007; Doghman et al., 2005). This is the first description of MC1R expression in human adrenal, and it will be interesting to determine what its specific physiological roles are in the human adrenal. One manner in which it may add value to the control of adrenal steroidogenesis and growth is that it differs from MC2R in having a physiological antagonist - agouti related peptide (AGRP) - which is itself expressed in the adrenal gland. AGRP is unregulated in Cushing's syndrome, and long term administration has been shown to inhibit steroidogenesis in mice, although these effects are not mediated through MC2R or MC4R. It is reasonable to propose that inhibiting MC1R signalling may be the means by which

AGRP influences adrenal steroid production. Improving our knowledge of this signalling pathway may open up avenues to new therapies for Cushing's syndrome - at present medical treatment is focussed mainly on the inhibition of steroidogenic enzymes directly.

ACTH and alpha-MSH bind to the MC1R with equal affinity, so it is not necessary to invoke alpha-MSH as a physiologically relevant endocrine hormone in humans. The absence of a significant intermediate lobe of the pituitary gland in adult humans would support this argument, but alpha-MSH immunoreactivity is present in the vast majority of human adrenal glands. By immunohistochemistry there is more alpha-MSH in the pars anterior than the pars intermedia, and it is also detected in the serum of patients with Addison's disease, mainly as desacetyl-alpha-MSH (Coates et al., 1988; Coates et al., 1986). The zona intermedia is more prominent in the fetal adrenal gland, and there is evidence for production of alpha-MSH and CLIP-like peptides in fetal extract (Silman et al., 1976). The pars intermedia is under tonic dopaminergic inhibitory control in rats, and the suggestion that this might also be the case in humans was provided indirectly in the observation that a dopaminergic antagonist increased alpha-MSH levels in pre-pubertal but not post-pubertal children (Facchinetti et al., 1995). A role for alpha-MSH or related intermediate lobe peptides in could be extrapolated from the temporal relationship between the presence of the FZ in the fetal adrenal, and its adult equivalent the ZR developing in pre-pubertal children. Interestingly, a recent Polish study found a statistically significant increase in skin pigmentation in 7-10 year old children (Sitek et al., 2013), so it would be interesting to measure serum alpha-MSH levels in more detail in this population. The physiological role for alpha-MSH in adrenal steroidogenesis has by no means been established definitively on the basis of the in vitro experiments described, where the applicability of pharmacological concentrations of alpha-MSH to the physiological setting is uncertain. However, the expression of adrenal MC1R provides a mechanism, and as noted above, one need not even invoke alpha-MSH for it to be important.

If one accepts the premise for a physiological alpha-MSH role in humans, it would be interesting to examine the stimuli for its release. Premature adrenarche in girls is associated with obesity and insulin resistance, and it has previously been hypothesised that the mechanism for this is the direct effect of insulin and insulin like growth factors (Güven et al., 2005). However, elevated levels of leptin have been demonstrated in these

patients, and leptin is a strong stimulator of alpha-MSH release (Keen-Rhinehart et al., 2013). It would be interesting to test the hypothesis that this unregulated alpha-MSH signalling is the mechanism by which insulin resistance leads to premature adrenarche. Furthermore, an improved mechanistic understanding of the dark skin pigmentation of acanthosis nigricans in insulin resistance would be welcome. Conversely, the long term effects of alpha-MSH agonists as tanning agents on the adrenal gland is yet to be elucidated (Langan et al., 2010). It would be interesting to see the effects of these biological agents on serum cortisol production, meanwhile the previously published case reports of Cushing's from a pure alpha-MSH secreting tumour remain a single case of a melanotroph adenoma in a feline patient (Meij et al., 2005)

Melanin was identified in the adrenal glands of patients with ACTH-dependent and independent Cushing's syndrome, along with markers of the melanosome organelle. The role for melanin in normal adrenal physiology is not clear, though as discussed it has an anti-oxidant and anti-inflammatory role in other tissues. It will be interesting to ascertain whether melanocytic markers and melanin are ubiquitous in the human adrenal gland - it is difficult to extrapolate from our tissue microarray data, given that although the tissue we looked at was not adenoma, the glands themselves were certainly not normal. A histopathological study of human adrenals removed at the time of nephrectomy would be straightforward.

The endocrine pedigree of gastro-intestinal stromal tumours has been predicted on the basis of expression of peptide hormones and synaptic vesicles necessary for their secretion (Bumming et al., 2007; Ekeblad et al., 2006). The data presented provide cautious support for this, although a full picture of the mechanism for GIST POMC processing and alpha-MSH secretion has not been provided. The identity of the POMC promoter used is not established, and there are reasons to doubt both the pituitary promoter due to its methylation status, and the peripheral promoter since its transcript would lack the signal sequence for ER targeting and secretion. The methylation status at all the CpG sites of the pituitary POMC promoter in the GIST tumour needs to be established, and a full sequence of the reported POMC transcript is required. As described previously, a 5' RACE approach would allow generation of at full length cDNA of the entire POMC transcript using primers at the 3' end (Yeku & Frohman, 2011), and confirm whether or not the signal sequence is included. This will be required to explain the absence of N-terminal

POC 1-28 and N-POC 1-49 immunostaining in the GIST, alongside positive alpha-MSH staining, and to demonstrate that immunostaining of any of these peptides can be thought to correlate with their secretion. There are some reports in the literature that tissues expressing almost exclusively the short 800nt POMC transcript are capable of generating a secreted peptide when stimulated to do so (Teofoli et al., 1999; Slominski et al., 2000), but these do not suggest a robust mechanism which gets around the problems outlined above. These papers consider human dermal fibroblasts in culture, so it would be valuable and fairly straightforward to attempt to replicate their experimental findings.

It is unlikely that alpha-MSH secretion from occult GIST tumours is responsible for a significant burden of disease, but it may be possible that a proportion of patients with the ectopic ACTH syndrome, or even subclinical Cushing's syndrome have a GIST that is not visible on routine imaging, and does not take up the radio labels commonly used to locate ectopic carcinoid tumours, for instance.

Making conclusions on the basis of initial studies on the roles of the CDK inhibitors in controlling rat adrenal remodelling were limited by technical issues related to immunostaining. With experience gained from the immunostaining of the adult human adrenal as described, it is planned to revisit that work. HMB-45 staining in the nucleus of the ZG in the human provided some indirect evidence that the cells are younger than those in the ZR, and it will be interesting to immunostain the adrenal tissue microarray in both normal and tumour samples for p27 and p57, since that is an extreme example of adrenal remodelling.

In conclusion, the data presented in this thesis suggest that POMC-derived peptides other than ACTH have a role in adrenal growth and steroid production. There is a potential that further elucidation of the mechanisms by which this occurs will improve our understanding both of adrenal neoplasia, and of adrenal insufficiency.

Chapter 7 References

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